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DORMANCY AND GERMINATION OF *ROTTBOELLIA EXALTATA* L. f.*

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ABSTRACT

The cause of dormancy of the seed of *Rottboellia exaltata* L. f. was studied together with some factors that influence its germination. Soil as germination medium promoted germination of intact seeds which is not attributable to microbial action or that of soluble substances in the soil. Exposure to light of soil-sown seeds gave a synergistic effect. Dehulling promoted germination but did not completely overcome dormancy. Exposure to light or sowing on soil almost completely removed dormancy of dehulled seeds. The application of gibberellic acid to dehulled seeds removed the light requirement whereas treatment with 20 ppm benzyladenine did not all promote germination. Water extracts of the hulls and of the intact seeds inhibited the elongation of the shoot of cucumber seedlings.

INTRODUCTION

Rottboellia exaltata is a tall grass belonging to tribe Andropogoneae and widely distributed as a weed in tropical and subtropical regions. It reproduces rapidly by profuse tillering and seed production. As much as 35 tillers are produced in 8 weeks and profuse branching can bring about as much as 546 inflorescence. Schwerzel[1] reported a total of 4,400 seeds produced by one Rottboellia plant.

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Newly mature seeds of Rottboellia exhibit dormancy and remain so for a year or longer.[2] Under field conditions it germinates in flashes, a characteristic which contributes to its being a noxious weed. Dehulling of dormant seeds has been reported to increase germination but not to completely break dormancy.[3] Crushing, pricking or partial dehulling resulted in improved germination. The restriction imposed by the hull was not overcome by treatment with 0.2% KNO_3, 100 ppm gibberellic acid (GA) or increased oxygen supply.

The study was conducted to determine the cause of seed dormancy in Rottboellia and identify some factors which may induce dormant seeds to germinate.

MATERIALS AND METHODS

Plant material. Mature Rottboellia seeds were handpicked from plants not previously treated with any herbicide. The term seed, as used in this study, refers to a single dispersal unit which may be pedicelled or sessile. Dehulled seed refers to the true seed liberated from the lemma and palea. The collected seeds were oven-dried at 41 ± 2°C for 7 hours, stored in paper bags inside a laboratory locker. Seeds stored in this manner gave 93-95% germination after a year when dehulled.

Germination tests. Immediately prior to sowing, seeds were surface sterilized with 2% calcium hypochlorite solution for 15 min and rinsed five times with sterile distilled water. In each experiment 50 seeds were sown in a Petri dish and germination counts were taken 4 days after. A seed with 2 mm radicle was considered germinated. All treatments were replicated 4 times.

Extraction and bioassay of the inhibitor. Thirty g of dried seeds were ground with 150 ml cold distilled water in a Waring Blendor for 30 min. The homogenate was cooled to 5°C and further homogenized for 3 min. The slurry was filtered through a sheet of coarse filter paper and the filtrate was centrifuged at 12,000 x g for 20 min at 10°C. The supernatant was brought to 90 ml with distilled water and used as inhibitor preparation. The inhibitory activity was tested with cucumber seeds.

RESULTS AND DISCUSSION

Germination in different media. Earlier laboratory studies on the germination of Rottboellia have been done with filter paper as medium and kept in the dark. Under such conditions

no germination could be obtained even after 4 months of storage. On the other hand, under field condition seeds which shed the current season could be observed to germinate even only after a month. To determine the reason for this discrepancy, seeds were allowed to germinate in different media in the dark. As shown in Table 1 highest germination percentage was obtained with soil as medium. When soil autoclaved for 20 min at 15 psi was used as germination medium, similar promotion of germination was obtained, ruling out the interference of microorganisms. The use of soil water extract in filter paper did not promote germination excluding water soluble components of the soil as responsible for the observed germination of otherwise dormant seeds. In *V. pyramidata*, Borriss [4] observed that the inhibitory substance in the seed is converted to a volatile metabolite which is adsorbed by the soil but not by filter paper.

TABLE 1. *Germination of* Rottboellia exaltata *in different media in the dark 65 days after storage in the greenhouse.*

Medium	*% Germination* [1]
Filter paper	0
Cotton	1
Sand	3
Soil—	
Non-sterilized	65.5
Sterilized	59.0

[1] Average of 3 replications, each with 50 seeds.

A similar case may happen in *Rottboellia exaltata*. An inhibitor which causes dormancy is leached out into the soil to a greater extent than in any other medium thereby promoting germination.

Effect of light on germination. Under field conditions germination higher than that obtained in the previous experiment has been repeatedly observed. One factor that can cause such difference is light. When seeds were sown on filter paper or soil and subjected to light or darkness, the data in Table 2 indicates promotion of germination by light. On filter paper, germination was increased as much as 9 times. On soil, promotion was more pronounced in seeds stored for shorter periods. The effects of soil and light in breaking dormancy appear synergistic.

TABLE 2. *Effect of light on the germination of* Rottboellia exaltata *stored for different periods in the laboratory locker.*

Length of storage at sowing (weeks)	PER CENT GERMINATION			
	Filter paper		*Soil surface*	
	Dark	*Light*	*Dark*	*Light*
1				1.0
2				4.0
3				8.0
4			1.0	10.5
5			2.0	18.0
6			3.0	24.5
7			8.0	44.0
8			7.0	49.0
9			10.0	56.5
10		1.0	11.0	57.0
11		2.0	10.0	53.5
12		3.5	13.5	55.5
13		2.5	15.5	65.5
14		3.0	15.5	59.5
15	0.5	2.0	18.0	71.0
16	0.5	8.0	18.5	77.5
17	1.5	11.5	21.5	83.5
18	3.0	22.5	19.5	83.0
19	3.5	25.5	14.5	85.0

The gradual loss of dormancy in seeds sown on soil but kept in the dark may represent the little by little leaching out of the inhibitor for the seeds and adsorbed by the soil. The effect of light in breaking dormancy may be due to overcoming inhibitor actions. Similar effects of light on dormant seeds have been observed in other species. The inhibitory effect of naringenin on the germination of lettuce seeds can be reversed by light.[5] Ikuma and Thimann,[6] on the other hand, observed that lettuce light caused the appearance of pectinase and celluase which presumably destroyed the restrictive integument. Light probably destroyed the inhibitor preventing the formation of such enzymes.

Effect of dehulling on germination. In the case of Rottboellia, the effect of light can not be that in the hull alone. As shown in Table 3 removal of the hull did promote germination but did not completely break dormancy, on observation similar to that in *A fatua.*[7] *A kotschyi,*[8] a *O. miliacea.*[9] Exposure of

dehulled seeds to light does overcome completely dormancy. Sowing dehulled seeds on soil removes the light requirement for germination. Thus, both soil as germinating medium and exposure to light effect the possible removal of the inhibitor controlling dormancy of dehulled seeds. The hull may impose a restriction to germination both as a physical barrier and through the action of an inhibitor.

TABLE 3. *Effect of dehulling on germination of* Rottboellia exaltata *on filter paper and soil under dark and light conditions.*

Treatment	PER CENT GERMINATION	
	Dehulled	*Intact*
FILTER PAPER		
Dark	31.5	0
Light	90.0	0
SOIL		
Dark	88.0	16.0
Light	90.0	71.0

Effect of GA and benzyladenine. In many species such as *S. lutescens* [10] *and S. syriaca,*[11] dormancy which is caused by an inhibitor, can be overcome by exogenous application of GA as shown in Table 4.

TABLE 4. *Effect of GA on germination of intact and dehulled* R. exaltata *seeds under dark and light conditions.*

Treatment	PER CENT GERMINATION			
	Intact		*Dehulled*	
	Light	*Dark*	*Light*	*Dark*
Control	0	0	67.0	21.0
GA—10 ppm	0	0	81.0	81.0
GA—25 ppm	0	0	89.0	88.0
GA—50 ppm	17.5	0	86.0	88.0
GA—100 ppm	19.5	0	89.0	87.0

When intact and dehulled Rottboellia seeds were treated with different concentrations of GA and exposed to darkness or diffused light GA can only promote germination of intact seeds

when exposed to light at higher concentrations giving 19.5 percent germination at 100 ppm. Removal of the hull made the seed totally responsive to GA treatment that dormancy was completely overcome at 10 ppm in the dark. GA has been shown to play a major role in the dormancy of *Avena fatua*.[12] Newly harvested seeds of this species did not produce GA but after 2.5 years, its dormancy period, GA was readily synthesized and dormancy was overcome completely. The failure of intact seeds to respond to GA in the dark may be due to the inability of GA to enter the embryo.

The action of light in the hull may allow partial disintegration of the hull through the action of hydrolytic enzymes as postulated by Ikuma and Thimann[6] in similarly photoblastic lettuce seeds. Such action would allow the entry of GA into the embryo and thereby promote germination.

When the seeds were slit at the distal end, treated with GA and kept in the dark, 20 percent germination was obtained at 100 ppm GA while only 4 percent germinated in the control. The radicle came out through the slit and appeared distorted. These observations indicate that the hull does impose a mechanical restriction on the embryo.

Exogenous application of benzyladenine at 20 ppm did not promote germination of either intact or dehulled seeds under light and dark conditions.

Growth-inhibitory effect of the water extract from intact Rottboellia exaltata seeds. As shown in Table 5 the water extracts from intact seeds stored for 4 months inhibited the elongation of the shoot of cucumber seedlings. There was no effect on germination itself. The growth inhibition induced by the extracts was more than 50 percent. No extract was taken from dehulled seeds due to the difficulty of accumulating enough materials. To prepare 10 ml of water extract 67 seeds were used. Considering the tremendous amount of seeds a plant can produce one season and shed to the ground where the inhibitor is leached, growth of other weed species will be suppressed and Rottboellia can easily become the predominant weed.

TABLE 5. *Effect of water extracts from intact seeds of* R. exaltata *on the germination and elongation of the shoot of cucumber.*

Treatment	*Per Cent Germination*	*Length of Shoot (mm)*
Water	96	40.7
Water extract	86	17.4

This and the previous observations all support the possibility that dormancy in Rottboellia is controlled by an inhibitor which may be present in the hull and in the seed. The production of numerous seeds together with the presence of an inhibitor in the seed which leaches out into the soil and suppresses other species contribute to the fast build-up of *Rottboellia exaltata* in many areas.

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OBSERVATIONS ON THE GROWTH OF YOUNG BANGOS, *CHANOS CHANOS* (Forskal) ON TWO TYPES OF PELLETED FOOD *

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ABSTRACT

Pre-fingerling, fingerling and juvenile bangos were seined from inshore Oahu (Hawaii) waters and raised in tanks on different pelleted foods to determine relative growths and mortalities, and the feasibility of totally artificial rearing. Collection efforts showed that young bangos occurred locally in shallow inshore waters. Bangos were graded into three size-groups (< 7, 7-11, > 11 g). Each group was divided into paired lots (700, 1000, 1150 g, respectively) that were placed in identical 0.56 cu m tanks with seawater flowing through 10 liters/minute. In two feeding trials, one series of fish was fed high-protein trout pellets (fish-meal base), the other was fed low-protein rabbit pellets (alfalfa base) costing only one-fourth as much as the former. Those fed with trout pellets showed higher weight gain. Also, mortality was lower among those fed with trout pellets (avg 8% vs. 16% for rabbit pellet diet). The surviving fish showed average weight gains of 27% (trout pellets fed) and 11% (rabbit pellet fed) in eight weeks. Diseases and temperature effects are considered. It is concluded that trout pellets are nutritionally superior than rabbit pellets under conditions of the experiment.

INTRODUCTION

The bangos, *Chanos chanos* (Forskal) is a large herring-like toothless fish. Its more distinct characteristics[1] are: an elongate body which is moderately compressed and pointed at both ends, a large caudal fin which is deeply forked, a broad and rounded abdomen, and a naked head with a large transparent imperforate adipose eyelid covering the eye and side of the head. Adults measure from three-fourths to one-and-a-half meters standard length and weigh from 11,900 to 18,600 grams. It is found throughout the warm portions of the Indian and Pacific Oceans, from southern Japan to Australia, and from southern California and Mexico to the Red Sea.[2]

* Condensed version of a directed research report submitted to the Hawaii Fishery Cooperative Unit of the Department of Zoology, University of Hawaii, in partial fulfillment of the requirements for the degree, M.S. in zoology.

Earlier studies on the feeding habits of the bangos had resulted in different conclusions. Seawater bangos have been variously classified as benthic feeders,[3] plankton feeders,[4] and both benthic and plankton feeders.[5] Estuarine bangos are herbivores [6] and brackish water bangos are benthic feeders when young [7] and herbivores when adults.[5] The disagreement is caused by divergent opinions regarding the origin of the food items and by the tendency of the bangos to facultative feeding.

The bangos is an important food fish in tropical Asia and is grown in commercial scale in India, Indonesia, Malaysia, Philippines and Taiwan. The practice is to catch the fry along the shallow portions of the seacoast and raise them up to 500 g in brackish water ponds. The mortality periods [5] connected with this practice are: at the fry stage, from 2 to 25 per cent during the shipment from the seacoast to the fishpond; at the fingerling stage from six to eight-week period at 50 to 70 per cent; from the juvenile stage up to a maximal 500 g weight in a period of eight to 10 months, from 20 to 30 per cent. The overall mortality is from 60 to 93 per cent. A mortality was estimated at 70 per cent from fry stage to marketable size.[8] It would benefit the bangos industry if the high mortality could be reduced. One way to reduce mortality is to raise the fry to juvenile in artificial environment which may then in turn, be stocked in ponds. This will give the fish a certain protection from natural enemies and a greater chance of survival by an increase in size.

Artificial rearing of young bangos involved seining selected inshore waters around Oahu for the fish and feeding controlled bangos with dissimilar pelleted foods. The objectives of this study are two-fold: first, to locate the best collecting grounds for young bangos and to determine when they occur inshore and second, to determine the feasibility of artificially rearing young bangos, demonstrate comparative growth and mortality on two pelleted foods.

The use of pelleted food for fishes had met considerable success as trout food in the United States,[9] carp food in Germany, and pike and trout food in Belgium.[10] At present, fish hatcheries in North America and Europe use pelleted food almost exclusively.

Pelleted food for fish production had not received similar attention in tropical southeast Asia and there was no published

literature on bangos raised with pelleted food. The growth of young bangos has been studied previously: in ponds[11] artificial feeding,[12, 13, 8, 5] and enriched diet.[14]

MATERIALS AND METHODS

In this study, young bangos were arbitrarily divided into three size-grades: pre-fingerlings are fish weighing 1.0-7.0 g; fingerlings, 7.1-11.0 g; and juveniles, 11.1-20.0 g. Based on Rabanal, et al., the pre-fingerlings, would be four to five weeks old; fingerlings, six weeks; and juvenile, seven to eight weeks.

Purina trout chow and rabbit checker pellets were used in the feeding experiments. The trout chow pellet contained 40.0 per cent protein while the rabbit checker pellet had only 15.5 per cent. Protein in trout pellet was from fish meal and that in rabbit pellet, from alfalfa meal. Enough pellets from the same shipment was bought at one time to last throughout the study.

Solutions of potassium permanganate ($KMNO_4$), pyridyl-mercuric acetate (PMA) and malachite green were used to control disease in the fish. Tricainemethane sulfonate (MS-222) solution was used to anesthetize fish during weighings.

Six aquaria, each measuring 0.8 x 0.5 x 1.4 m (56 cu m capacity), were used. Seawater continuously flowed into and out of each aquarium at an average of 10 liters per minute. Each aquarium had a bottom drain and a wire-screen cover that prevented fish from jumping out.

Other equipment consisted of an Ohaus platform balance with a capacity from 0.1 g to 5.0 kg, a scoop net, a rubber siphon, and mercury thermometer.

Acclimation. — Fish were transported from collecting grounds to the Hawaii (State) Division of Fish and Game laboratory at Kewalo Basin in Honolulu in a fiber drum aboard a truck. At the laboratory, aerators were added and seawater was allowed to drip into the drum to gradually bring water to the temperature and salinity of the laboratory seawater. This lasted from two to four hours, depending upon the difference in temperature and salinity of the water from which the fish and the laboratory seawater were taken. Fish were then transferred to a holding tank and fed with trout pellets. Acclimation to pellet feeding took two days.

Feeding. — Each size-grade of fish (see Materials) was divided into two equal lots and placed in adjacent aquaria. One lot per size-grade was fed with trout pellets and the duplicate, with rabbit pellets. The trout pellet ration was four per cent of body weight of the fish while the rabbit pellet ration was six per cent. Half of the daily ration was given in the morning and half in the afternoon. Two sets of feeding experiments were performed, each lasting two months.

Weighing. — Fish were weighed at the start of the experiment and every second week thereafter. To prepare fish for weighing, the aquaria was drained except for about five centimeters of water and MS-222 was added to anesthetize the fish. The fish were removed with a scoop net, placed in a previously weighed bucket with clean water and weighed on a platform balance. Most of the fish recovered from the anesthetic during weighing. The rest recovered in the experimental aquaria. Total time that fish were out of the aquarium was from 10 to 12 minutes. The difference between the first and second weighings was taken to be the weight of the fish.

RESULTS AND DISCUSSION

Collecting sites. — Biological collections were made around Oahu from June to September at 14 sites (Fig. 1) to determine the best source of young bangos. The fish were found in only four sites (closed circles, Fig. 1). Further samplings in the four sites showed that young bangos were present only from September through December. Bangos used in this study came from the Paiko Tidal Flat on leeward Oahu and the Molii Pond on the win windward side.

Growth on pelleted food. — A total of 292 fish were fed with trout pellets and 291 were fed with rabbit pellets. Growth is expressed as per cent of body weight increase from initial weight. Results showed that fish on trout pellet diet gained 27 per cent in eight weeks while those on rabbit pellet gained only 211 per cent. Analysis of data according to fish size showed that in all three size-grades, growth was better in fish on trout pellet diet (Fig. 2). The effect of diet is more apparent in the pre-fingerlings as shown in the wide gap between lines 5 (trout pellet diet) and 6 (rabbit pellet diet).

There are no data in published literature with which results in this study could be compared. In nursery ponds described by Rabanal, et al. (1952), five-week bangos (equivalent to pre-

fingerlings in this study(increased 21 per cent in two weeks, a value that is higher than the 16 per cent obtained for pre-fingerlings for the same two-week time. Data were further analyzed to determine whether different size-grades exhibited different growth rates. Results (Fig. 3) showed that although fingerlings gained the least weight on both diets, the fastest weight gainers on rabbit pellet diet were the pre-fingerlings while the fastest gainers on trout pellet diet were the juveniles. These do not show clearly the effect of size upon growth although earlier workers found size to be a factor. [11,8] Brown, [15, 16] found that growth rate in small individuals increased when removed from a mixed-size population.

Disease. — Only a few incidences of disease were observed. One cause was tail rot as manifested in the erosion of the caudal fin. The whole experimental lot in which the infected fish was observed was treated with $KMNO_4$ (1:100,000 dilution) or PMA (1:500,000) solution. No study to determine what caused tail rot in the bangos was made but in gold fish (*Carassium auratus*), it was due to bacteria of the genera Aeromonas and Pseudomonas.[17]

Injured fish, i.g., fish with missing scales, were susceptible to fungus infection. This disease was successfully treated with any of these solutions: $KMNO_4$, PMA or malachite green (1:100,000 dilution). No attempt was made to identify the fungus in the experimental fish. Leitritz[18] reported *Saprolegnia parasitica* in trout and salmon and Nozal', Vashchenko and Tarasova[19] controlled Saprolegnia in pike eggs with malachite green (1:200,000 to 1:100,000 dilution).

A third disease, the "whitening of one or both eyes", was observed in some newly collected juveniles which impaired feeding. Petrushevskii[20] reported a similar disease in trouts as "helminthic cataract of the eye" caused by the metacercariæ of *Diplostomulum spathaceum.* As a result of the destruction of the visual epithelium, the fish became blind but if the parasites died or were encapsulated, the fish would have recovered normal vision. In this study, the disease was eliminated with the addition of PMA (1:500,000 dilution) for two days.

Mortality. — Another criterion for the effect of diet is mortality. Subject to similar conditions, a better diet should

result in lower mortality. Mortality according to diet and size-grade are shown in the Table 1.

TABLE 1. *Mortality based on diet of young bangos.*

Diet	PER CENT MORTALITY		
	Pre-fingerling	*Fingerling*	*Juvenile*
Trout pellet	7	5	13
Rabbit pellet	20	12	16

Mortality in fish on trout pellet diet (8 per cent) was only one-half of that in fish on rabbit pellet diet (16 per cent). For those on trout pellet diet, juveniles suffered the highest mortality (13 per cent) while those on rabbit pellet diet, pre-fingerlings incurred the highest mortality (20 per cent). For both diets, fingerlings suffered the lowest mortality.

Artificially rearing of young bangos reduced mortality to a significant extent as found in this and earlier studies. In this study, mortality was lower than the 50 to 70 per cent given by Schuster (1960) for fingerlings raised in fishponds. Earlier studies on smaller specimens (bangos fry from one to three weeks old) showed that mortality in artificially reared fish were also lower (11 to 23 per cent, Ronquillo and Villamater, 1957; 4 to 31 per cent. Ronquillo, et al., 1957) than the 50 to 70 per cent by Ronquillo and Villamater (1957) for fish under pond cultivation.

Growth and water temperature. — Analysis of data showed no effect of water temperature on growth under the conditions in this study. This was expected since temperature fluctuated only between 23.8°C and 24.1°C. Where temperature range is larger, e.g., 3°C, metabolic differences are obtained.[21]

SUMMARY

Young bangos which occur in the inshore waters of Oahu between September and December can be artifically reared on either trout pellet or rabbit pellet. Growth in fish on trout pellet diet was more than twice with that in fish on rabbit pellet diet. In the sizes studied, size as a factor affecting growth was not conclusively demonstrated. Water temperature was stable and

did not affect growth. Growth rate of artificially reared fish was significantly lower than fish reared in ponds.

Incidences of diseases (tail rot and fungus infection) in the experimental fish were few. A third disease, the "whitening of the eye", was observed only in some newly collected juveniles.

Mortality in fish on rabbit pellet diet was twice more than in fish on trout pellet diet. Artificial rearing of young bangos significantly reduced mortality.

On the basis of higher weight gain and lower mortality, trout pellet is a better food for young bangos than rabbit pellet.

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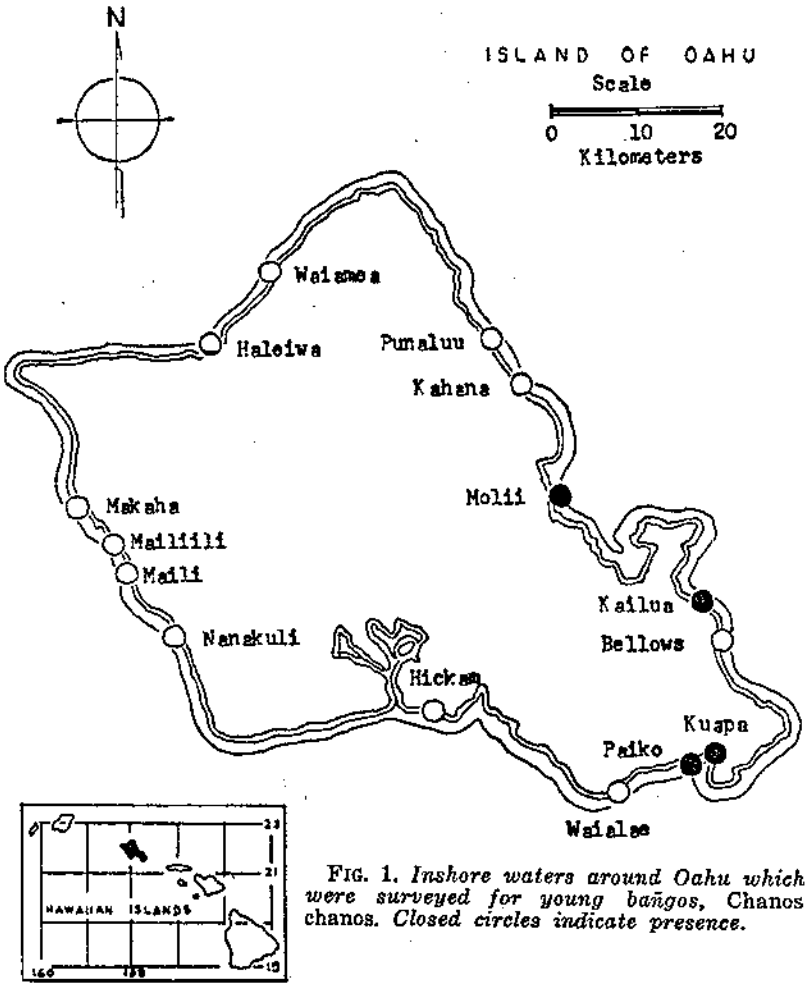

FIG. 1. *Inshore waters around Oahu which were surveyed for young bañgos,* Chanos chanos. *Closed circles indicate presence.*

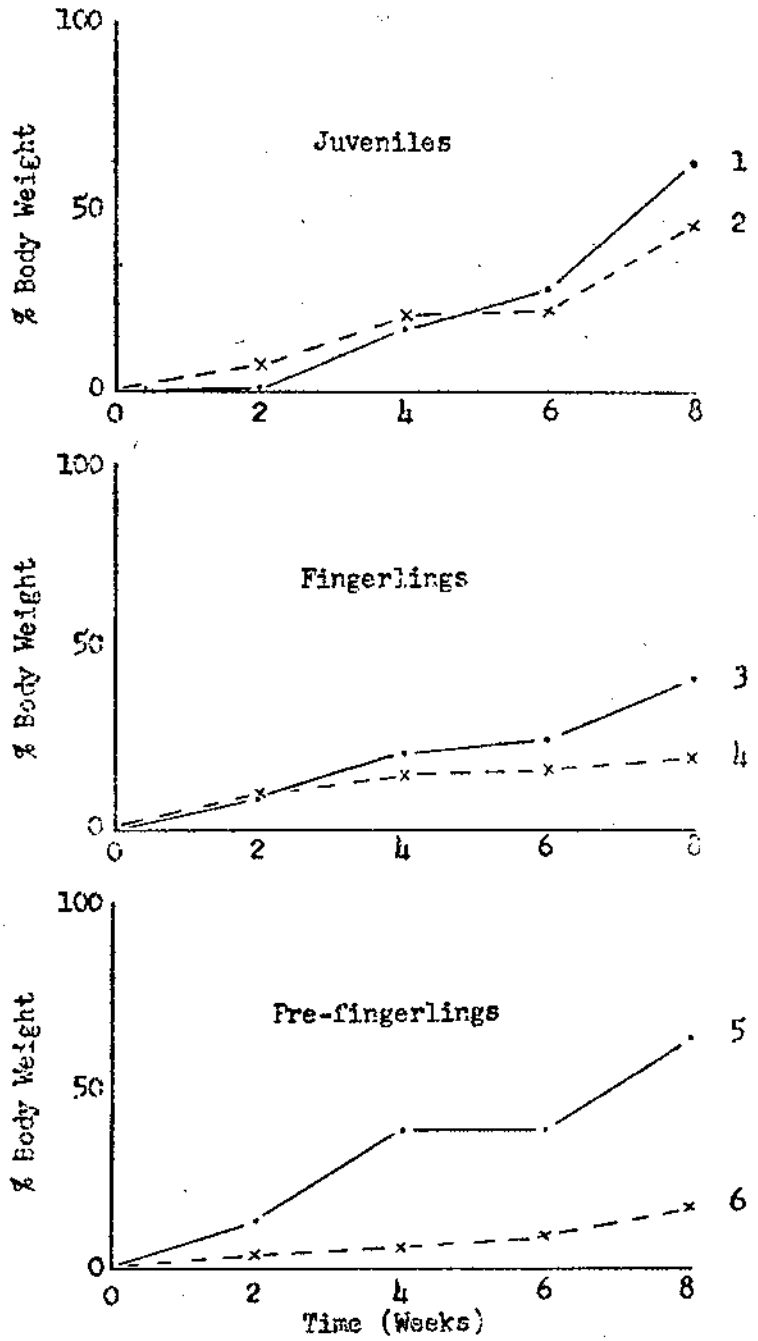

FIG. 2. *Bi-weekly growth of bañgos on two types of artificial food according to size-grade. Note that in all size-grades, weight gain of fish on trout pellet diet was greater than of fish on rabbit pellet diet. (.——. trout pellet diet, x——x rabbit pellet diet).*

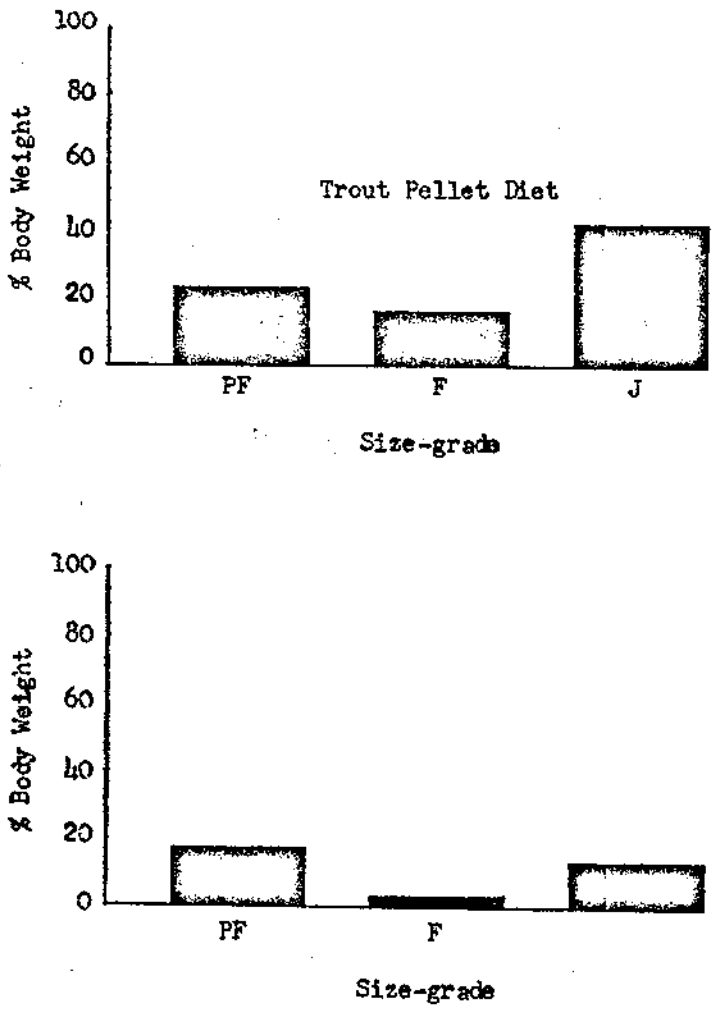

FIG. 3. *Growth of young bañgos on two types of artificial food according to size-grade. Note that the weight-gain of fish on trout pellet diet was more than twice that of fish on rabbit pellet diet (PF = pre-fingerlings, F = fingerlings, J = juveniles)*

VITAMIN B_{12} AND ANTIBIOTIC ACTIVITIES OF ACTINOMYCETES ISOLATED BY A SELECTIVE METHOD

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ABSTRACT

A total of 249 streptomycetes were obtained by selective isolation from 171 soil samples collected from various parts of the country. These were grown in solid and liquid media and tested for their vitamin B_{12} and antibiotic activities.

In solid medium, 64 isolates were found to produce vitamin B_{12} with growth zones ranging from 8.5 to 22.0 mm and in liquid media, 143 were active, producing 1 to 120 ug vitamin B_{12}/100 ml fermentation brew.

One hundred sixty-six isolates showed antibiotic activity in solid medium and 94, in liquid media. Of the former, 14 were active against all test organisms; 136, against *Bacillus subtilis;* 119, against *Micrococcus pyogenes* var. *aureus* 209P; 96, against *B. cereus* var. *mycoides;* 70 against *M. pyogenes* var. *aureus* (penicillin resistant); 53, against *Escherichia coli;* and 22, against *Pseudomonas aeruginosa.* Of the latter, two were active against all test organisms; 77, against *B. subtilis;* 68, against *pyogenes* var. *aureus* 209P; 60, against *B. cereus* var. *mycoides;* 49, against *M. pyogenes* var. *aureus* (penicillin resistant); 9, against *E. coli;* and 5, against *Ps. aeruginosa.*

Some isolates were active when grown in the solid medium but lost such activity when grown in the liquid media. Changes in the composition and state of the culture medium seemed to influence the vitamin B_{12}-and antibiotic-producing capacities of the isolates.

The actinomycetes are a group of organisms which occur naturally in the soil and in other habitat. They are involved in various biological processes in the soil. Some of those belonging to the genus *Streptomyces* are known to produce antagonistic substances and vitamin B_{12}.

The possibility of isolating actinomyces from their natural habitat in order to reduce or eliminate bacterial and fungal contamination and favor the development of actinomycete colonies has been reported. In the study of the microbial popu-

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lation of acid-forest soils, Corke and Chase[1] found that the addition of 40 ug/ml of cycloheximide to the culture medium inhibited fungal growth effectively.

Dulaney *et al.*[2] isolated Streptomyces on a nutrient agar medium containing the following antibiotic combinations: cycloheximide, polymixin, and penicilin. Williams and Davies[3] incorporated four antibiotics; namely, nystatin, actidione, polymixin B sulfate and, sodium penicillin into starch plus casein medium and successfully achieved selective growth of actinomycete colonies from the soil. Porter *et al.*[4] reported that pimaricin, nystatin, and cyclohemimide suppressed molds on isolation plates. These antibiotics were also found to be effective in decontaminating mold-infested streptomyces cultures.

Media with glycerol or starch as the carbon source and nitrate and casein as the nitrogen sources were found to be suitable for the selective growth of streptomyces colonies over other organisms.[5]

Rickes et al.[6] reported *Streptomyces griseus* as a new source of vitamin B_{12}. Since then, considerable interest has been aroused in the production of the vitamin by streptomycetes and other microorganisms.[7, 8, 9, 10]

In the Philippines, antibiotic-producing streptomyces were isolated by Sevilla-Santos and de Leon[11] and Sevilla-Santos and Bernardo,[12] using ordinary plate dilution method without the addition of any antibiotics as inhibiting agents.

This paper reports the isolation of 249 actinomycetes from 171 soil samples as a result of the selective isolation method. It also gives the antibiotic and vitamin B_{12} activity of the above isolates grown in solid and liquid culture media.

MATERIALS AND METHODS

Source of soil samples.—Soil samples used in the selective isolation of the streptomyces cultures were collected from various parts of the country.

Media used.—The composition of the different media is presented in Appendix A.

Test organisms.—For the detection of antibiotic activity, the following bacteria were used as test organisms: *Micrococcus pyogenes* var. *aureus* 209P, *M. pyogenes* var. *aureus* (penicillin resistant), *Bacillus subtilis, B. cereus* var. *mycoides, Escherichia coli,* and *Pseudomonas aeruginosa.*

For the determination of the vitamin B_{12} activity, a mutant strain of *E. coli* was used.

Selective isolation method.—Soil dilutions of 1:100, 1:1000, and 1:10000 were prepared. One ml from each dilution was placed in each of three sets of three sterile petri dishes per soil sample. Three antibiotics, namely: chlortetracycline, oxytetracycline and, tetracycline (2 mg/ml) were used as treatment, one antibiotic for each set of petri dishes at 0.5 ml per plate. Nystatin (2 mg/ml) was added to all plates at 0.5 ml per plate. Eight ml of melted starch casein agar (45-50°C) was added to each plate and the contents mixed thoroughly. The agar was allowed to solidify, the plates inverted and incubated at room temperature for 3 to 7 days. Characteristic streptomyces colonies which grew were transferred into Emerson agar slants.

Production of Vitamin B_{12} and antibiotic substances by the streptomyces isolates. — The methods used by Sevilla-Santos and Bernardo[12] in the cultivation of the isolates was followed except that in the present study the shake cultures were sampled on the *3rd*, *5th*, and *7th* days of incubation.

Testing for vitamin B_{12} activity. — The procedure of testing for vitamin B_{12} content was by the microbiological plate method of Harrison *et al*[13] which is briefly stated as follows. A loopful from the stock culture of mutant *E. coli* was streaked into culture maintenance agar and incubated at 27° to 29°C for 18 to 24 hours. The 24-hour growth of *E. coli* was inoculated in peptone water medium and incubated at the same temperature for 18 to 24 hours. The seeding inoculum was prepared by diluting the organism with peptone water medium until it gave the turbidity when compared to a suspension giving 65 per cent light transmission.

The assay plates were prepared as follows: Ten ml of molten assay medium, which is the base layer, was poured and allowed to solidity in the assay plates. To 200 ml of assay medium, 20 ml of sterile 13 per cent dextrose solution was added. The medium was then allowed to cool to 47°C in an accurately regulated water bath and a 1 per cent of the seeding inoculum was added. Three and a half ml of this seed layer was overlayed on the base layer of each assay plate and allow to solidify.

Sterile paper discs (6.5 mm) which were dipped in the fermentation brew or agar chunks (7.0 mm) of the streptomyces under test were placed on the agar seeded surface. The plates

were incubated at 27° to 29° for 18 to 24 hours after which the diameter of the zones of exhibition or growth stimulation around each agar chunk or paper disc was measured. The vitamin B_{12} content in the liquid media was read from a standard vitamin B_{12} curve using dilutions of 0.3, 0.25, 0.2, 0.15, 0.01, and 0.04 ug/ml.

Testing for antibiotic activity. — The antibiotic activity of the isolates which were grown in solid and liquid media, was tested by the agar plug and the paper disc methods, respectively, as described by Sevilla-Santos and Bernardo.[12]

Rectangular Pyrex glass trays (7″ × 12″) which accommodated 120 assay samples were used instead of Petri dishes. A base layer was prepared by pouring 100 ml of melted Emerson agar into the sterile tray and allowing the agar to solidify. Over the solidified base layer, 50 ml of melted Emerson agar at about 45°C was seeded. The test organisms to be used were streaked in nutrient agar slants and incubated for 24 hours at 37°C. Spore suspensions of *B. subtilis* and *B cereus* var. *mycoides,* prepared by following the procedure of Grove and Randall,[14] were used at 65 per cent light transmittance, while the other test organisms at 20 per cent.

Sterile paper discs (6.5 mm) which were dipped into each sample for the liquid media tests and agar plugs (7.0 mm diameter) for the solid media tests were carefully placed on the surface of the seeded agar in glass Pyrex trays. The trays were incubated at 37°C for 18 to 24 hours and the antibiotic activity of the cultures was determined by measuring the zone of inhibition around each disc or chunk.

RESULTS AND DISCUSSION

A total 249 streptomycetes was obtained by selective isolation from 171 soil samples which were collected from different parts of the country. The sources of soil and the corresponding number of cultures isolated from them are given as follows: Batangas, 70; Nueva Ecija, 45; Rizal, 40; Cavite, 32; Laguna, 33; Pampanga, 17; Corregidor, 11; Tarlac, 6; Iloilo, 8; Bulacan, La Union and Zambales, 3 each; and Manila, 1.

Vitamin B_{12} — active streptomyces isolates. — Results of the screening of streptomyces isolates in solid and liquid media for vitamin B_{12} activity are summarized as follows:

No. of isolated tested in solid medium 249
No. of active isolates 64
No. of inactive isolates 185
No. of isolates tested in liquid media 249
No. of active isolates 143
No. of inactive isolates 106

In solid medium, 64 streptomycetes (26 per cent) were found to be active producers of vitamin B_{12} with growth zones ranging from 8.5 to 22.0 mm. In liquid media, 143 (57 per cent) were active, producing 1 to 120 ug per 100 ml. The higher percentage of isolates showing vitamin B_{12} activity in liquid media shows that if the promising isolates are grown in a suitable medium, higher vitamin B_{12} production might be attained. The semisynthetic nature of the liquid media may also explain the result obtained due to the presence of substances which are stimulatory to the production of the vitamin.

Of the 64 active isolates, 56 (88 per cent) gave exhibition zones ranging from less than 8.5 to 12.4 mm, and 8 (13 per cent) gave zones of growth from 12.5 to 22.0 mm. The latter eight promising isolates and the corresponding zones of growth produced are shown in Table 2, Shete *et al*[9] reported that 56 (28 per cent) of their 200 cultures gave growth zones above 13 mm. Results in the present study indicate that 8 (12 per cent) out of the 64 active isolates gave growth zones greater than 13.0 mm.

TABLE 2. *Vitamin B_{12} activity of eight promising streptomyces isolates in solid medium.*

Isolate Number	*Exhibtiion Zones (mm)*
S-70-14	22.0
S-70-5	20.0
S-70-13	18.5
S-70-23	18.0
S-70-66	15.0
S-70-33	13.5
S-70-127	12.7
S-70-37	12.5

Results of the present study show that in culture broths, 143 isolates produced vitamin B_{12} ranging from 1 to 120 ug/ 100 ml. A grouping of the vitamin B_{12} activity of the cultures on the 5th day of incubation is presented in Table 3.

TABLE 3. *Grouping of streptomyces isolates according to the vitamin B_{12} produced in four different culture media.*

Content	NUMBER OF ACTIVE ISOLATES			
(ug/100 ml)	*Med. I*	*Med. II*	*Med. B*	*Med. D*
1-40	85	67	92	39
41-80	3	1	3	1
81-120	3	4	7	—
TOTAL	91	72	102	40

Table 4 shows the activity of 25 isolates considered promising. The highest yields were obtained when the isolates were grown in medium B, although there was noticeable variation in the growth response from culture to culture. There were isolates which produced as much as 100 ug/100 ml of vitamin B_{12} in medium B, but were not able to produce any amount in the other media. Isolates S-70-98, -127, and -123 gave vitamin content of 100 ug/100 ml, while isolates S-70-99, -102, -119, and -120 produced from 100 to 120 ug/100 ml.

Antibiotically active streptomyces isolates. — A summary of the results of the screening of streptomyces isolates in solid and liquid media for antibiotic production are as follows:

No. of isolates tested in solid medium 249
No. of active isolates 166
No. of inactive isolates 94

No. of isolates tested in liquid media 249
No. of active isolates 94
No. of inactive isolates 155

Of the 249 isolates tested in solid medium, 166 (66.27 per cent) showed antibiotic activity of varying capacities against at least one of the six test organisms used, while 84 (33.73 per cent) were nonactive. When the same isolates were tested in liquid media, 94 (37.75 per cent) were found to possess antibiotic properties, while 155 (62.25 per cent) did not show any activity against any one of the test organisms.

The distribution of streptomyces isolates producing varying degrees of antibiotic activity against at least one of the six test organisms, in solid and in liquid media is presented in Table 5. In solid medium, 136 isolates or 55.02 per cent were active against *B. subtilis,* 119 or 47.79 per cent against *M.*

pyogenes var. *aureus* 209P, 96 (39.10 per cent) against *B. cereus* var. *mycoides* and, 70 (28.11 per cent) against *M. pyogenes* var. *aureus* (penicillin resistant). Fifty-three isolates or 21.88 per cent were found to be active against *E. coli* and, 22 isolates representing 8.93 per cent were inhibitory to the growth of *Ps. aeruginosa*. Only fourteen isolates or 5.64 per cent inhibited the growth of all the test organisms in solid medium.

In liquid media, 94 isolates (37.75 per cent) inhibited the growth of at least one test organism. Seventy-seven isolates (31.56 per cent) were active against *B. subtilis*, 68 (27.30 per

TABLE 4. *Vitamin B_{12} production of streptomyces cultures after five days of cultivation in four liquid media.*

Isolate number	Vitamin B_{12} Content (μg/100 ml)			
	Medium I	*Medium II*	*Medium B*	*Medium D*
S-70-127	+++	+	+++	—
S-70-118	++	+	+++	—
S-70-119	++	++	+++	—
S-70-114	+++	+	+	—
S-70-124	+++	+	+	—
S-70-101	++	—	+++	++
S-70-123	+	+	+++	+
S-70-130	+	+	++	+
S-70-133	+	+	++	+
S-70-102	—	+	+++	+
S-70-103	+	—	+++	—
S-70-98	—	+	+++	—
S-70-135	+	+	++	—
S-70-120	—	—	+++	—
S-70-99	—	—	+++	—
S-70-121	—	+	+++	—
S-70-81	+	+	+	+
S-70-131	+	+	+	+
S-70-53	+	+	+	—
S-70-126	+	+	+	—
S-70-138	+	+	+	—
S-69-126	+	+	+	—
S-69-33	—	+	+	—
S-70-134	+	—	+	—
S-70-110	—	+	—	—

Legend:
+ = 1 to 40 μg/100 ml
++ = 41 to 80 μg/100 ml
+++ = 81 to 120 μg/100 ml

TABLE 5. *Number of streptomyces producing varying degrees of antibiotic activity in solid and liquid media.*

Zone of Inhibition (mm)	*Number of Isolates Active Against*											
	M. progenes aureus 209P		*M. pyrogenes aureus* (R)		*B. subtilis ATCC* 6633		*B. cereus* var. *mycoides*		*E. coli*		*Ps. aeruginosa*	
	S	*L*	*S*	*L*	*S*	*L*	*S*	*L*	*S*	*L*	*S*	*L*
27 and above	1	4	2	—	4	6	—	—	—	—	—	—
21 to 26	17	4	13	4	15	7	17	6	—	—	—	—
15 to 20	29	16	26	14	58	20	33	19	13	2	10	2
14 and below	72	44	29	31	59	44	46	35	40	7	12	3
Total	119	68	70	49	136	77	96	60	53	9	22	5
Per Cent	47.8	27.3	28.11	19.70	55.02	31.56	39.10	24.1	21.28	3.68	8.93	2.0

Number of isolates tested — 249
S — Solid media
L — Liquid media

cent) against *M. aureus* 209P, 60 (24.10 per cent) against *B. cereus* var. *mycoides*, and 49 (19.70 per cent) against *M. aureus* (penicillin resistant). Nine isolates (3.68 per cent) were active against *E. coli* and, 5 (2 per cent) against Ps. aeruginosa. Only two isolates were active against all test organisms in liquid media.

Among the test organisms used, *E. coli* and *Ps. aeruginosa*, both gram-negative bacteria, were the most resistant to the antagonistic action of the streptomyces isolates. Moreover the percentage of cultures active upon the Gram-positive bacteria only was higher than that of strains active upon either both Gram-positive and Gram-negative bacteria, or upon the Gram-negative bacteria only. The above findings confirm earleir reports made by other investigators that the Gram-negative bacteria, especially *Ps. aeruginosa* exhibit some degree of resistance to almost all antibiotic agents. Similar findings have earlier been reported by Burkholder,[15] Chun,[16] Landerkin *et al.*,[17] Johnstone,[18] and Emerson *et al.*[19]

Changes in the composition and state of the culture medium seemed to influence the antibiotic-producing capacity of the isolates. Some isolates were active in solid medium but lost or refused to produce such activity when grown in liquid media. The reverse case was also true. Likewise, there were more isolates which showed inhibitory effects in solid medium than in liquid media. It may be assumed that the variations in the ingredients and in the composition of the culture media affected the capacity of the isolates to produce antibiotic substances. According to Waksman,[20] some isolates are capable of producing a volatile substance which is toxic to other organisms. It is also possible that actinomycetes form substances different from ammonia, but the production of these compounds can probably explain the discrepancies between the inhibitory effects observed when the actinomycetes are grown in solid and liquid media.

SUMMARY

Two hundred forty-nine streptomyces isolates were grown in solid and liquid media and tested for vitamin B_{12} production and antibiotic activity.

In the test for vitamin B_{12} production of the isolates, 26 per cent were found to be active in solid medium with growth zones ranging from less than 8.5 to 22.0 mm. In liquid media, 57 per cent produced vitamin B_{12} ranging from 1 to 120 ug/100 ml.

One hundred sixty-six, or 66.67 per cent of the isolates tested in solid medium exhibited antagonistic properties of varying capacities against at least one of the six test organisms used. In liquid media, 94 (37.75 per cent) showed inhibitory effects, while 155 were inactive.

The predominant antibiotic spectrum of activity of the isolates both in solid and in liquid media was against *B. subtilis* and *M. pyogenes* var. *aureus* 209P.

ACKNOWLEDGMENTS

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APPENDIX A

I. Composition of media used in the isolation of Streptomyces Cultures (g/1)

1. Starch casein agar

Starch	10.0
Bacto-casein	0.3
Potassium nitrate	2.0
Sodium chloride	2.0
Potassium phosphate (dibasic)	2.0
Calcium carbonate	0.02
Magnesium sulfate	0.05
Ferrous sulfate	0.01
Agar	20.0
Distilled water	1000 ml
pH 7.0	

2. Emerson agar

Dextrose	10.0
Bacto-peptone	4.0
Beef extract	4.0
Yeast extract	1.0
Sodium chloride	2.5
Distilled water	1000 ml
Agar	20.0
pH 7.0	

II. Composition of media used in assay for vitamin B_{12} content

1. Culture maintenance medium

Acid-hydrolyzed casein	6.00
Di-potassium hydrogen phosphate	0.20
Ferrous sulfate	0.005
Magnesium sulfate	0.2
L-asparagine	0.15
Glycerol	2.0
Vitamin B_{12}	400 ug
Distilled water	1000 ml
Agar	20.0
pH 7.2 $\pm$ 0.1	

2. Peptone water medium

Peptone	10.0
Sodium chloride	2.5
Distilled water	1000 ml
pH 7.2 $\pm$ 0.1	

3. Assay medium

Prepare the stock solutions A, B and C

Stock solution A

Ammonium chloride	100.0
Ammonium nitrate	40.0
Distilled water + 0	1000 ml

Stock solution B

Potassium dihydrogen phosphate	20.0
Dipotassium hydrogen phosphate	60.0
Distilled water + 0	1000 ml

Stock solution C

Sodium borate	0.094
Copper sulfate	0.250
Ferrous sulfate	0.540
Manganese chloride	0.460
Zinc sulfate	4.900
Ammonium molybdate	0.020
Distilled water + 0	1000 ml

Add hydrochloric acid dropwise until the solution is clear (approximately 0.5 ml is normally required). These stock solutions should be stored at 4°C and should be removed every two months.

Medium

Stock solution A	250 ml
Stock solution B	250 ml
Stock solution C	0.5 ml
5 per cent w/v magnesium sulfate	10.0 ml
Asparagine	7.5 g
0.1 per cent calcium chloride	5.0 ml
Distilled water + 0	4.6 l
Agar	52.5 g
pH 7.2 ± 0.1	

APPENDIX B

Composition of culture media used in the selective screening (g per l)

Ingredients	*Med. I*	*Med. II*	*Med. B*	*Med. D*
Dextrose	10	10		
Corn starch	10	10	5	20
Soy beans	15		30	
Cornsteep liquor (50 per cent solids)				10
Beef extract		7.5		
Bacto-peptone		7.5		
Bacto-casein			1	
Ammonium nitrate				5
Potassium phosphate (dibasic)	1			
Magnesium sulfate	0.5			
Sodium nitrate			3	
Calcium carbonate			5	4
Sodium chloride	3	3		2
pH	7.2	7.2	7.0	6.8

Media I, II, and B were formulated in the Laboratory of the Antibiotics Section. Medium D was obtain from Belgovskaya and Popova, J. Gen. Microbiol, **20** (1959), 462.

MICRODETERMINATION OF FOLIC AND CHROMOTROPIC ACIDS

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ABSTRACT

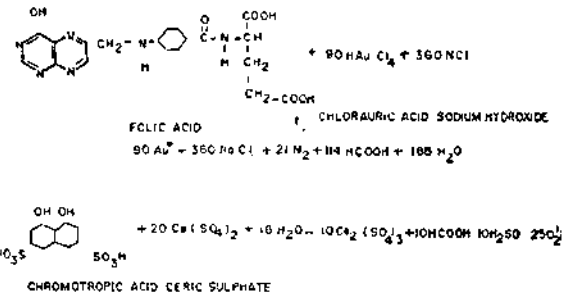
Folic and chromotropic acids have been quantitatively determined in micro amounts by oxidizing with chlorauric acid in the presence of large excess of alkali and with ceric sulphate in highly acidic medium, respectively. In the case of folic acid the reaction was completed at 45 equivalence; while in the case of chromotropic acid the reaction required 20 equivalence to complete. Excess alkali in the case of folic acid and excess sulphuric acid in the case of chromotropic acid gave absurd results. Maximum error in both cases was 1.2 per cent. Both methods are reliable and reproducible.

INTRODUCTION

The literature towards the determination of folic acid is not very rich in that only a few titrimetric methods have been mentioned. However, folic acid has been determined by a modification of Baker's method nephlometrically;[1] polarographically in multivitamin preparations;[2] colorimetrically;[3, 4] potentiometrically by titration of the reduced (diazotized) and non-reduced (nitrosated) forms of folic acid with $NaNO_2$ in dil. HCl;[5, 6] microbiologically;[7, 8, 9, 10] by a method based on the cleavage by reduction in an acid solution to yield petridine and an aromatic amine, the amount of aromatic amine was determined by Bratton and Marshall's method and is used as a measure of pteroyl derivative present;[11] by coulometric method or by titration with chloramine T solution;[12] chromatographically;[13] by the modification of Mitchell & Snell in which Lactobacillus casei is used and the growth response was measured by determining the turbidity of the medium spectrophotometrically;[14] enzymically;[15] by Konig reaction;[16] titrimetrically;[17] and fluorometrically.[18]

No particular literature is available for the determination of chromotropic acid. Both folic and chromotropic acids find their use in a number of processes but no attempt has been made to find out a simple quantitative methods for their determination.

In order to find out a simple quantitative method for their determinations, the present work has been proposed. Present work deals with the quantitative determination of folic and chromotropic acids in micro amounts by oxidizing with chlorauric acid in alkaline medium and with ceric sulphate in a very highly acidic medium, respectively. In the reaction with chromotropic acid, ceric sulphate has been used as oxidizing agent in the presence of acid, and the equilibrium is established at 20 equivalence. In the reaction between folic acid and chlorauric acid in the presence of large excess of lakali, the oxidation is completed at 45 equivalence. The following reactions take place:



The formation of formic acid in both cases has been detected and the confirmation of which has been discussed in detail.

MATERIALS AND METHODS

Reagents used. — Folic acid and N-phenylanthranilic acid (B.D.H. grade); gold chloride or chlorauric acid (Apex, Indian grade); ferrous ammonium sulphate, potassium ferrocyanide, sodium carbonate, sodium bicarbonate and sulphuric acid (ANALAR B.D.H. grade); sodium hydroxide and chromotropic acid (E. Merck grade); ether solvent (Alembic grade); and ceric sulphate (Technical B.D.H. grade).

Micropipettes and microburettes used have least count of 0.01 ml. Hot plate with regulated heat control system and Whatmann No. 42 filter papers were used.

Folic acid solution (2.0×10^{-4}M) was prepared by dissolving an exactly weighed amount in 0.01N NaOH solution.

Chromotropic acid solution (1.0×10^{-3}M) was prepared by dissolving an exactly weighed amount in distilled water.

Standard ferrous ammonium sulphate solution was prepared by dissolving an exact amount in 0.01N H_2SO_4 solution.

N-phenylanthranilic acid of 0.1 g and 0.2 g Na_2CO_3 were mixed together and dissolved in 20 ml distilled water by vigorous shaking. It was transferred to 100 ml volumetric flask and the volume was raised up to the mark by adding distilled water.

Ceric sulphate solution (3.2×10^{-3} M) was prepared in 4N H_2SO_4 solution and standardized by titrating against standard ferrous ammonium sulphate (in 0.01N H_2SO_4) solution using N-phenylanthranilic acid solution as indicator.

Potassium ferrocyanide solution (10.5×10^{-3}M) was prepared by dissolving an exact amount in distilled water and which was further standardized against standard ceric sulphate (in 4N H_2SO_4) solution using the above mentioned indicator.

Chlorauric acid solution (14.4×10^{-3}M) was prepared in distilled water and further standardized.[19]

Microdetermination of folic acid by oxidizing with gold chloride in alkaline medium. — Known volumes of standard folic acid solution were taken in different beakers, through micropipette, and known excess of standard chlorauric acid and sodium hydroxide solutions were also added. With constant stirring, 50 ml distilled water was added which formed the solution mixture. Beakers containing solution mixture were placed on a hot plate at full heat (keeping in mind that the solution mixture may not evaporate) for 60 minutes. In case the volume of the reaction mixture reduced to 10 ml, much before 60 minutes, 20 ml more distilled water were added. The reaction mixture was cooled at room temperature after scheduled heating, filtered, washed with distilled water; acidified the filtrate and the washings with 20 ml 2N H_2SO_4 solution, and then a known excess of standard potassium ferrocyanide solution was added. Remaining ferrocyanide solution was titrated by running through a microburette against standard ceric sulphate (in 4N H_2SO_4) solution using N-phenylanthranilic acid solution as indicator. At the end point, a reddish brown color appeared sharply.

Microdetermination of chromotropic acid by oxidizing with ceric sulphate solution. — Known volume of standard chromotropic acid solution was taken in a beaker, through micropipette, and known excess of standard ceric sulphate (in 4N

H_2SO_4) solution along with 30 ml distilled water was added — which formed a solution mixture after stirring. The reaction mixture was placed on a hot plate at full heat for 150 minutes, keeping in mind that the reaction mixture may not evaporate. In case the volume of the reaction mixture in the beaker reduced to 10 ml, much before 150 minutes, 20 ml more distilled water was added. The reaction mixture was cooled at room temperature and then the remaining ceric sulphate solution was titrated against standard ferrous ammonium sulphate (in 0.01N H_2SO_4) solution using N-phenylanthranillic acid solution as indicator. At the end point, pink red solution vanished sharply with the appearance of pale yellow color.

Results are given in Tables 1 and 2. The range in which folic and chromotropic acids have been estimated vary from 17.55×10^{-3} mg to 44.50×10^{-3} mg; and from 7.21×10^{-2} mg to 17.98×10^{-2} mg, respectively.

The reaction between folic acid and gold chloride in the presence of large excess of sodium hydroxide results in the rupture of the molecule requiring 22.5 oxygen atoms and in the formation of formic acid as main oxidation product. In the case of the reaction between chromotropic acid and ceric sulphate in highly acidic medium, the rupture of the molecule required 10 oxygen atoms resulting in the formation of formic acid as oxidation product and sulphur dioxide gas evolved.

As the oxidation product being the same, i.e., formic acid, in both folic and chromotropic acids but in different medium, hence, the reaction mixture, in the case of folic acid, was acidified and the presence of formic acid was confirmed separately according to the following scheme:

The reaction mixture in acid solution was extracted with ether and washed. The etherial extract was shaken with a saturated sodium bicarbonate solution and the bicarbonate extract was acidified with 8N H_2SO_4 and extracted with ether. The ether extract was washed until free from sulphate ions and then concentrated. The concentrate gave positive tests for formic acid and the identity of which was confirmed by co-chromatography on paper.

Since the reaction gets completed between folic acid and chlorauric acid in large excess of alkali at 45 equivalence, calculations have been done by dividing the titer values by 45;

TABLE 1. *Microdetermination of folic acid with chlorauric acid.*

Folic acid 0.0002M ml	NaOH 0.025N ml	$HAuCl_4$ 0.0144M ml	$K_4Fe(CN)_6$ 0.0105M ml	$Ce(SO_4)_2$ 0.0032N ml	$Ce(SO_4)_2$ 0.0032N ml	Amount of folic acid Taken x 10^3mg	Found x 10^3mg	Per cent error
—	—	—	3	9.64	—	—	—	—
—	—	2	3	0.64	9.00	—	—	—
0.2	4	2	3	1.20	0.56	17.64	17.55	0.5
0.3	4	2	3	1.49	0.85	26.46	26.64	0.6
0.4	4	2	3	1.78	1.14	35.28	35.73	1.2
0.5	4	2	3	2.06	1.42	44.10	44.50	0.9

TABLE 2. *Microdetermination of chromotropic acid with ceric sulphate.*

Chromotropic acid 0.001M ml	$Ce(SO_4)_2$ 0.0032N ml	$FeSO_4(NH_4)_2SO_4$ 0.005N ml	$FeSO_4(NH_4)_2SO_4$ 0.005N ml	Amount of chromotropic acid Taken/Found $\times 10^2$mg		Per cent error
—	10	6.44	—	—	—	—
0.2	10	5.63	0.81	7.82	7.21	1.2
0.3	10	5.24	1.20	10.68	10.68	0.0
0.4	10	4.82	1.62	14.24	14.42	1.2
0.5	10	4.42	2.02	17.80	17.98	1.0

and in the case of chromotropic acid calculations have been done by dividing the titer values by 20. It has been observed that large excess of alkali in the case of folic acid gave absurd results. One of the necessary precautions with these experiments is that inadequate heating may also be responsible for absurd results. This is a simple method which is reproducible and gives concordant values. Results show a maximum error in cases of folic and chromotropic acids at 1.2 percent.

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PRODUCTION OF PROTEOLYTIC ENZYME I. EFFECT OF IRRADIATION ON PROTEASE PRODUCTION BY *ASPERGILLUS ORYZAE* (AHLBURG) COHN.

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ABSTRACT

The Philippine strain of *Aspergillus oryzae* (Ahlburg) Cohn, was exposed to ultra-violet rays and ionizing radiation from Cobalt-60 for the purpose of obtaining possible mutants or resistant strains which produce powerful proteolytic enzymes. Out of 58 isolates, only 3 gave significant proteolytic values (PV) high enough to merit further investigation. The isolates were picked from plates exposed to gamma-rays from Cobalt-60.

INTRODUCTION

Enzymes are among the most useful industrial products elaborated in the living cells of plants, animals and microorganisms. Even in small amounts they are capable of accelerating chemical reactions that are utilized in the continuous breakdown and buildup of cells, digestion of foods, conversion of sugar into alcohol, tenderizing meat, cheese making and in many other biological processes. They belong to a class of complex proteins which are able to take up foreign proteins, carbohydrates and fats, to break these up into simpler compounds and to rebuild them into the particular ingredients that make up the living protoplasm. The early knowledge on enzymes arose from studies on fermentation brought about by microorganisms. As knowledge about enzymes accumulated, scientists learned their technical uses as well as their practical applications and had succeeded in "training" microorganisms to produce the right kinds of enzymes in industry.

Several fermentation industries are dependent on microbial enzymes. There are great possibilities that available enzyme

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products can be harnessed for industrial purposes in the Philippines such as in the leather manufacturing, food and feed industries, in the preparation of pre-digested foods, for hydrolyzing fish and plant proteins into palatable sauces, as detergent in the laundry industry and for several medicinal preparations.

The *Aspergillus* molds are among the best sources of industrial enzymes. Prescott and Dunn[1] reviewed the industrial applications of enzymes of the *Aspergillus flavus-oryzae* group which are extensively used in Japan and other countries in the manufacture of fermented soybean products, alcoholic liquors and in food processing. Hepner and Male[2] described some important outlook in the future of these enzymes particularly in cosmetics and pharmaceuticals. A very popular enzyme recently introduced in the Philippines is the one now used as laundering detergent to facilitate removal of obstinate stains.[3,4,5,6] Fungal proteolytic enzymes have also been utilized in the extraction and clarification of fruit juices.[7] Crude fungal enzyme supplement have already been established as important feed additives.[8,9,10,11,12]

In the Philippines, Baens-Arcega *et al.*[13] isolated a species of *Aspergillus oryzae* whose proteolytic enzyme was three times more efficient than the most active strain studied by Oshima and Church in 1923.[14] Based on the cultural and morphological characteristics of the organism, it was identified as a typical strain of *A. oryzae,* which is well-known for its enzyme-forming ability, by Drs. Dorothy I. Fennel and C. W. Hesseltine of the Agricultural Research Service, U.S. Dept. of Agriculture, Peoria, Illinois. It was lyophilized by ATCC as culture No. A-5777.

Temperature and pH studies conducted by Baens-Arcega *et al.*[15] in 1966 revealed that the protease of the local isolate may be used for enzymatic processes at a temperature not higher than 50°C with pH values between 6.0-9.5. Its capacity as a valuable hydrolyzing agent in some industrial processes has been demonstrated in several studies.[16,17]

Microorganisms, like any other living bodies, are subject to undergo changes in their biochemical and physiological activities depending upon conditions in their environment. The recent advances in Microbiology, particularly microbial genetics, have provided a means of improving the efficiency of many fermentation processes resulting in a quantitative increase in

yields of products or synthesis of new compounds. The effect of ultra-violet and ionizing radiations on some mold species have already been reported.[18,19,20] In the recently concluded Symposium in Vienna, Austria in 1971, results of studies on radiation and radioisotopes for industrial microorganisms were given prominence in the topics of reports.[21] Irradiation is a means of improving the efficiency of many fermentation processes. The enzyme-forming capacity of the Philippine isolate may, therefore, be enhanced through radiation mutation. According to Calam[22] the main objective in strain improvement program is to increase productivity to commercially feasible level; the best strain available must be obtained which is then improved by mutation and selection.

In this study it is hoped that by irradiation, a potentially high enzyme-yielding mutant will be isolated and its physiological properties investigated to determine optimum factors for protease production.

MATERIALS AND METHODS

The Philippine strain of *A. oryzae* (A-5777) was exposed to ultra-violet and ionizing radiations to isolate possible mutants which can produce potent proteolytic enzymes for use in various industries. Following radiation treatments cell suspensions were serially diluted and plated in Czapek's agar (Raper and Fennell[23]). The isolates which survived different radiation exposures were picked and tested for efficiency. Based on the number of colonies on the plates after incubation for 5 days under room conditions, a survival count was plotted against time of exposure for both treatments.

Preparation of samples to be irradiated. — The local isolate (A-5777) was rejuvenated in freshly prepared Czapeck's agar medium. A 7-day-old agar slant culture was used to make a spore suspension of approximately 10^7/ml with the use of a Neubauer Haemocytometer before exposure to ultra-violet light. A UVS.11 mineralight lamp with a wavelength of 2537°A was used in this study. Spore suspensions were placed in open petri dishes approximately 150 mm beneath the UV lamp and each exposed at varying length of time from 2 to 23 minutes at 3-minute intervals. Exposures were done in a dark chamber to prevent possible photoreactivation of the spores after treatment.

Three-day-old sporing colonies in separate petri dishes were exposed for 1 hour to gamma rays from Cobalt-60 at different doses — 10, 30, 50, 70, 90, and 110 kilorads to determine the dose that will produce a 99% kill.

Plating and isolation of the surviving colonies. — Following exposure to radiation treatments, cell suspensions were serially diluted and plated on Czapek's agar to yield individual, well-separated colonies. The plates were stored in the culture chamber for five days to determine the survival count. Typical colonies were picked off and transferred onto agar slants. The isolates obtained from said plates were used for the screening programme.

Preparation of inoculum.—Ten grams of rice bran were moistened uniformly with 8 ml of water in 250-ml Erlenmeyer flasks. The flasks were plugged with cotton and autoclaved at 15 psi for 20 minutes. The cooled, sterilized bran medium was seeded with spores of the irradiated isolates and incubated at its optimum temperature range at 24-27°C. A three-day old rice bran culture of the organism was used as inoculum in all succeeding experiments.

Preparation of mold medium.—Ten grams of copra meal was placed in 250-ml Erlenmeyer flasks. To each flask was added 12 ml of water and stirred thoroughly to moisten the materials uniformly. The flasks were plugged with cotton and autoclaved at 15 psi for 20 minutes. The sterilized media were sufficiently moist to allow even distribution of inocula and uniform growth of the fungus.

Screening of the possible mutants for protease production.— Each isolate was grown separately in copra meal medium for 3 days at 24-27°C and afterwards macerated with 88 ml of water for 3 hours to extract the enzyme. The extracts were strained through cheesecloth and then filtered repeatedly through ordinary filter paper until clear.

The procedure adopted by Baens-Arcega *et al.* was used for the assay of the enzyme product. Ten ml of the filtered aqueous extract computed to contain the proteolytic enzyme produced from 1 gram of the fungus culture was diluted with distilled water to a volume of 100 ml. Portions of this enzyme solution such as 0.1, 0.2, 0.3, 0.4, 0.5 ml, etc. were allowed to digest 5

ml of a 0.5% casein solution in separate test tubes. The digestion was carried on for 1 hour at 40°C. The appearance of cloudiness or precipitate after adding 0.5 ml of a mixture of saturated magnesium sulfate and concentrated nitric acid (4:1) to the digestion tubes indicate incomplete digestion, the density of cloudiness or precipitate being dependent upon the amount of undigested casein. The clear tube next to the one showing opacity is taken as the tube containing the minimum amount of enzyme which digested completely the casein in the 5 ml of a 0.5% solution.

To express the proteolytic activity the following unit was used: If 0.025 g (or ml) of the original enzymic substance digest completely 5 ml of 0.5% casein solution (0.025 g casein) in 1 hour at 40°C, then the proteolytic value (PV) of this substance is 100.

Selection of the most promising isolates.—The proteolytic activity of each isolate was determined separately and the isolates with the highest proteolytic value were selected and used in the succeeding experiments. Stock culture of the selected possible mutants were preserved under mineral oil and in soil medium to preserve the characteristics of the organism.

Influence of incubation temperature on protease production.—To determine the optimum temperature for protease production by the selected isolates after irradiation treatments, 40 flasks containing copra meal medium were separately inoculated with spores of the irradiated culture and incubated at different temperatures, 23-25°C, 24-27°C, 28-30°C, 33-34°C, and 35-37°C. Duplicates were provided for each treatment. The PV and percentage yield of the enzymes produced were determined quantitatively 3 days after incubation.

Determination of the effect of incubation period on protease formation.—Fifty flasks containing copra meal medium were inoculated with the irradiated spores and incubated at the temperature optimum for the growth and activity of the isolates, 24-27°C. The PV and percentage yield of the enzymes produced by the irradiated cultures were then determined daily for a period of 7 days. Duplicates were provided for each determination. Crude enzyme was precipitated from the aqueous extract by the addition of 95% ethyl alcohol to give a final concentration of 70%. The precipitate was dried to constant weight in a Precision oven at 45°C and weighed.

This research paper deals principally with screening of irradiated isolates for proteolytic activity using a convenient and practical method of Oshima and Church.[14] The crude enzyme powder produced by the selected isolates will be evaluated more quantitatively with the use of an Autoanalyzer. Results will be published in the second part of this paper on "Some factors affecting enzyme formation by irradiated *Aspergillus oryzae*."

RESULTS AND DISCUSSION

Colony count of the surviving organisms.—The number of surviving organisms after exposure to ultra-violet and ionizing radiations is shown in Fig. 1. The graph shows a decrease in the number of survivors as exposure time was prolonged. Assuming 100% as the initial colony count, the number decreased to 90% when exposed to ultra-violet light for 2 minutes, 36% when exposed for 5 minutes, 20% survived after 8 minutes exposure, 12% after 11 minutes, 4.0% after 14 minutes, 1.8% after 17 minutes, 0.6% left after 20 minutes and 0.20% survived after 23 minutes exposure. Eight typical colonies were obtained from ultra-violet irradiated spores.

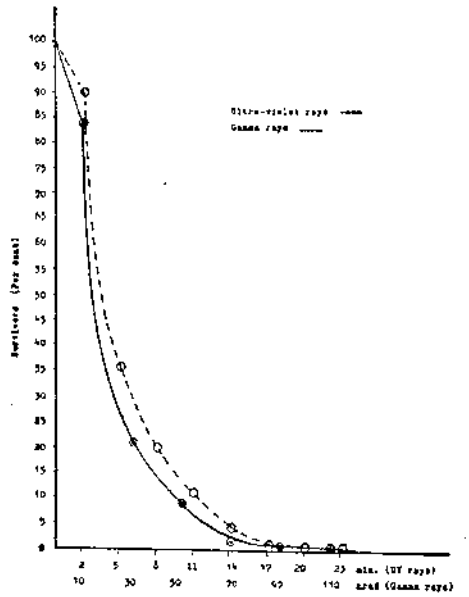

FIG. 1. *Survival curve of the Philippine strain of* A. oryzae *after exposure to ultra-violet and ionizing radiations.*

A very similar death curve was observed when the parent mold was exposed to ionizing radiation. Approximately 83.5% survived the first exposure at 10 kilorads gamma-rays, 21% survived the second exposure at 30 kilorads. The number of survivors decreased significantly to 8.6% at 50 kilorads, went down to 1.3%, 0.4% and finally to 0.021% as the dose was increased to 70, 90 and 110 kilorads, respectively. As expected, a 99% kill was obtained in both treatments at the end of the process. Eight typical colonies were picked and screened for proteolytic activity.

To test the effectivity of double radiation exposure, first to ultra-violet rays and then to ionizing radiation, as a means of improving the efficiency of the organism, UV-irradiated isolates were exposed to gamma-radiations from Cobalt-60 at different doses. After serial dilutions and platings, forty-two isolates were selected and screened for proteolytic activity. It is believed that the surviving colonies obtained from the processes of irradiation may be possible mutants or resistant strains capable of producing more potent enzymes when cultured under optimum conditions.

Screening of the possible mutants for protease production.— A total of fifty-eight isolates which survived different irradiation treatments were screened for protease-forming ability. Ultra-violet rays gave maximum proteolytic values of 357 only as compared to the parent strain (A-5777) which gave a proteolytic value of 500, showing no improvement over the parent strain. It is possible that ultra-violet rays are weak mutagenic agents when applied to the Philippine mold to alter the genetic properties and physiological activities of the organism favorable for enzyme production.

Two gamma-irradiated isolates, numbered G-10 and G-110, showed significant increases in proteolytic activity over the parent strain giving values of 833 and 625 respectively. Low enzyme-yielding UV- and gamma-irradiated isolates were discarded.

Forty-two doubly-irradiated spores were screened for proteolytic activity. Among these isolates, only one, labeled 23-110, gave a high proteolytic activity, PV 625; the remaining isolates, with proteolytic values from 277 to 568 were discarded.

The three highest enzyme-yielding isolates, G-10, G-110, and 23-110 were therefore selected for the study of some factors conducive for the production of proteolytic enzymes.

Influence of incubation temperature on protease production.—The effect of incubation temperature on PV of the enzymes produced by the selected isolates are presented in Fig. 2-A. All the irradiated isolates exhibited their peak of proteolytic activity, PV 625-833, when incubated at 24-27°C. This temperature range conforms with the findings of Baens-Arcega *et al.*[15] in their work on proteolytic enzyme. However, lower temperatures, 23-25°C, may still be favorable for the cultures under study as shown by their considerably high activity, PV 625. The activity of the isolates decreased significantly as the temperature of incubation was raised. Among the isolates, 23-110 gave the highest activity, PV 833 when incubated at temperatures between 23-27°C.

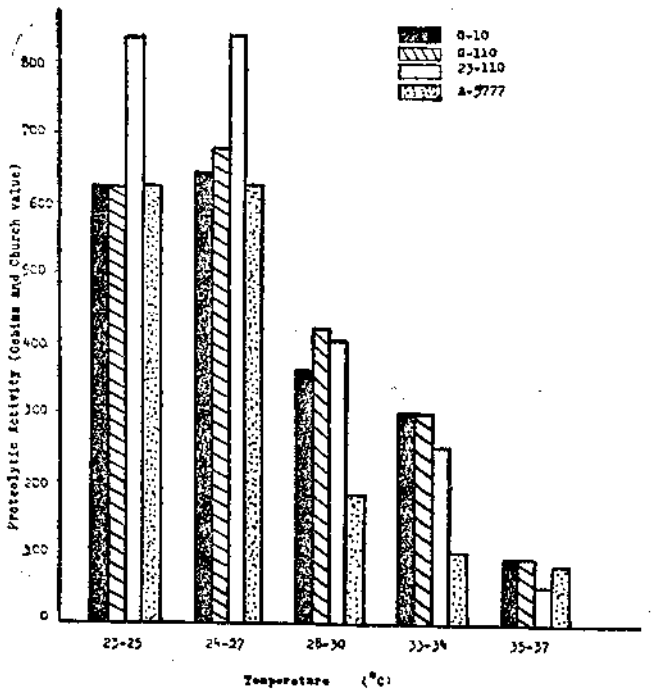

FIG. 2-A. *Effect of incubation temperature on pv of enzyme produced by the selected isolates.*

The enzyme yield of the organisms when incubated at different temperatures are plotted in Fig. 2-B. The active enzyme was precipitated from the clear filtrate with 95% ethyl alcohol. No marked differences in the yields were observed at higher temperatures of incubation probably because enzymes have the property of retaining their primary structures even if denaturation is involved. The relationship between enzyme yield and activity appears insignificant when incubated at optimum temperature of 24-27°C. Isolate G-110 gave the highest PV, 677, with an average yield of 10.44% enzyme. The parent organism, A-5777, gave a slightly lower PV of 625 and an average yield of 10.55% enzyme which is slightly higher. Similarly, isolate G-10 gave a higher PV of 646 than A-5777 but with a lower enzyme yield, 9.33%.

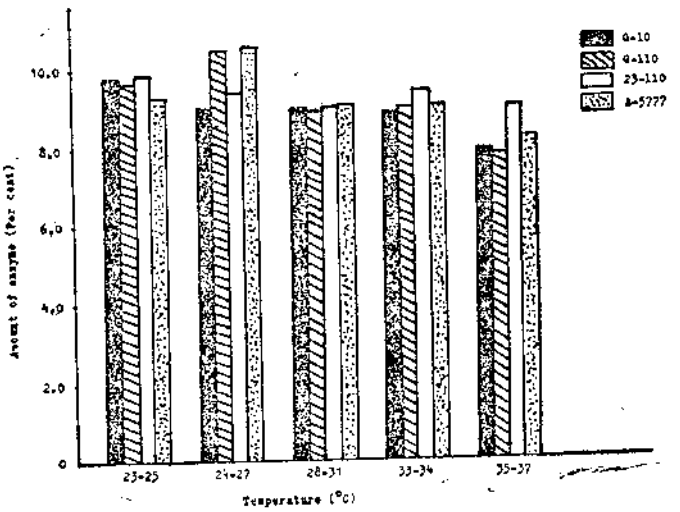

FIG. 2-B. *Effect of incubation temperature on yield of enzyme of the irradiated isolates.*

Determination of the effect of incubation period on protease formation.—The relationship of incubation period to enzyme activity of the irradiated organisms under study are shown in Figs. 3-A to 3-D. It appears that all the organisms formed the enzyme in their cells as early as the second day when the spores are still young and immature, and retained considerable potency up to the fifth day of incubation. For isolate G-10, a very low activity, PV 83, was obtained on the first day of incubation, Fig. 3-D. The activity sharply increased

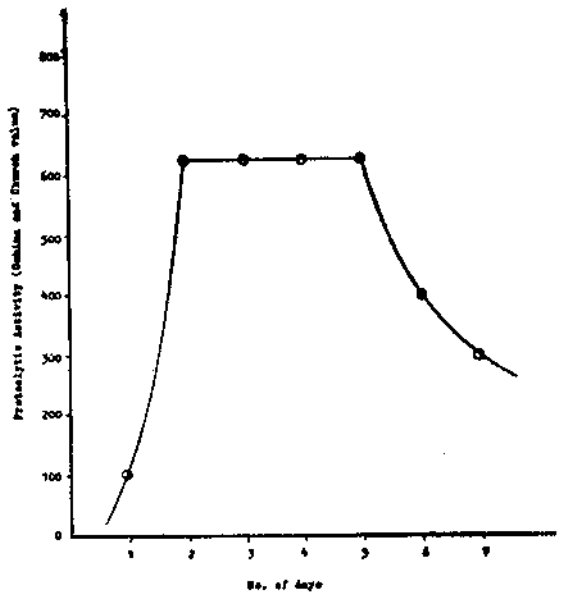

FIG. 3-A. *Effect of incubation peirod on pv of isolate G10.*

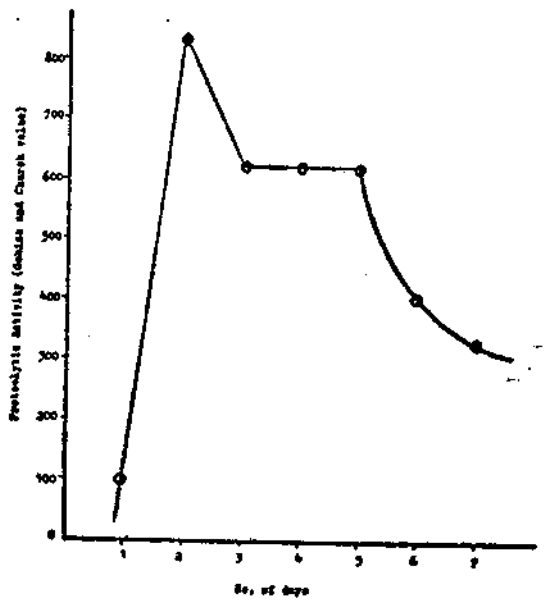

FIG. 3-B. *Effect of incubation period on pv of isolate G-110.*

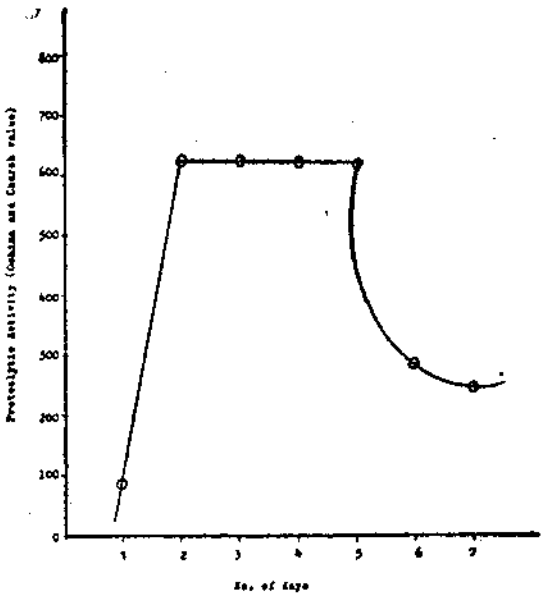

FIG. 3-C. *Effect of incubation period on pv of isolate 23-110.*

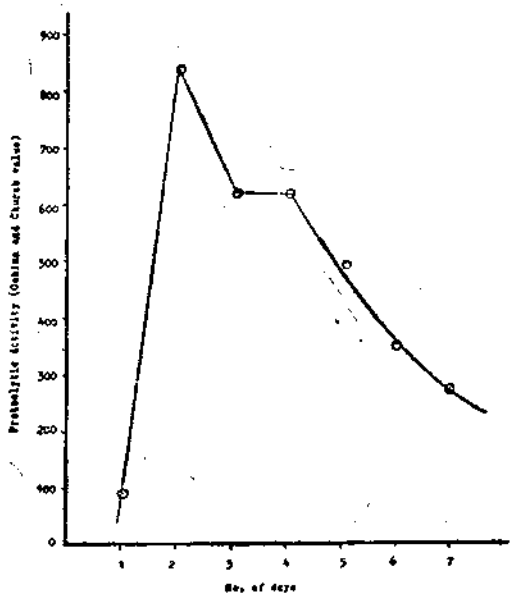

FIG. 3-D. *Effect of incubation period on pv of isolate A-5777.*

to PV 625 on the second day, maintaining its activity up to the fifth day of incubation, and then decreased. Figure 3-B shows the activity curve of isolate G-110. It can be noted that this isolate reached also its peak of activity on the second day but was more active than isolates G-10 and 23-110 having a PV of 833. Considerable potency was maintained up to the fifth day of incubation and then gradually declined.

Activity curve (Fig. 3-C) similar to Fig. 3-A was observed for isolate 23-110 which gave highest activity, PV 625, from the second day up to the fifth day of incubation.

Figure 3-D shows the activity of the parent mold, A-5777. It has a high PV, 833, identical to isolate G-110 and higher than isolates G-10 and 23-110. The activity of the parent organisms, however, declined after the fourth day whereas the irradiated cultures maintained a high level of activity up to the fifth day. This behavior suggests that 2-day-old cultures of the selected irradiated strains were as active as 5-day-old cultures. Enzymatic activity of all strains decreased after the fifth day of incubation.

Quantitative determination of enzyme yields (Fig. 4) revealed that all the isolates produced maximum amounts of the enzyme on the second day of incubation. The maximum enzyme yields of each isolate were 9.5% for G-10; 9.6% for G-110; 9.16% for 23-110 and 10.07% for the parent mold. This ob-

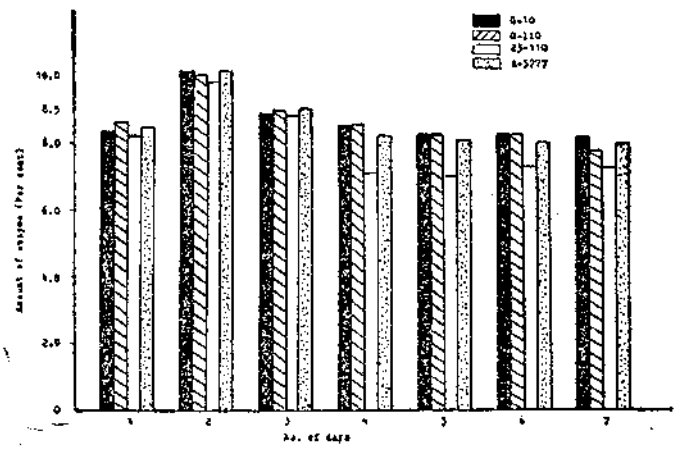

FIG. 4. *Comparative amounts of enzyme produced by the irradiated calories for a 2-day period of incubation.*

servation revealed that active enzyme-forming strains gave more proteolytic enzymes.

It had been reported in previous studies (Baens-Arcega *et al.*)[13, 15] that maximum enzyme production occurred after three days of incubation. The present study reveals, however, that enzyme formation can take place as early as the second day. The difference could be a manifestation of the possible mutants isolated from the parent strain.

Investigations conducted by Meyrath *et al.*[24] using irradiated *A. oryzae* for amylase production showed no marked differences from the parent strain with respect to the time needed to reach maximum enzyme production.

SUMMARY

The Philippine strain of *Aspergillus oryzae* (Ahlburg) Cohn. was exposed to ultra-violet rays and ionizing radiation from Cobalt-60 for the purpose of obtaining possible mutants or resistant strains which produce powerful proteolytic enzymes.

Out of 58 isolates, only 3 gave significant proteolytic values comparable to, or even better than the parent strain. The isolates, numbered G-10, G-110 and 23-110 were picked from plates exposed to gamma-rays from Cobalt-60.

Optimum incubation temperature for the three isolates was shown to be between 24-27°C giving highest percentage of active protease.

The isolates were found capable of producing active protease from the second day of incubation up to the fifth day, an improvement over the parent strain whose activity was retained up to the fourth day only, although no marked difference was observed in the percentage yield of the enzyme produced by each isolate.

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EMBRYOGENESIS IN *AMARANTHUS SPINOSUS* LINN. AND *AMARANTHUS VIRIDIS* LINN.

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ABSTRACT

Embryogenesis of *Amaranthus spinosus* and *A. viridis* was followed from the club-shaped structure to the mature stage. Increase in size and length of the embryo was due to periclinal and anticlinal divisions. Intense cell activity is found in the apical meristems resulting in the formation of the shoot and root apices. Differentiation of cells starts in the spherical stage when the outer layer of cells forms the protoderm. The heart-shaped structure shows the beginnings of the procambium and cotyledonary initials. The hypocotyl-root axis starts to form also at this stage. In the torpedo stage, the ground meristem gradually takes form extending from the root tip to the cotyledons. The mature embryo with the two cotyledons show distinctly the three primary meristems — the protoderm, the procambium, and the ground meristem.

INTRODUCTION

A study of the formation of the embryo reveals the origin of the vegetative parts of the plant and the inception of tissue organization.[1] This serves as a starting point to the study of the structure of adult plants. It can also serve as a basic reference on embryogeny of a dicot species for Philippine schools, since the material used is easily available throughout the country.

In embryogenetic studies, the initial concern is with the growth and differentiation of a single cell, the zygote, which through development ultimately gives rise to the embryo. An embryo consists of an axis, the hypocotyl-root axis, bearing the root apical meristem at one end and the cotyledons and the shoot apical meristem at the other end.[2] Sometimes a shoot bud or the epicotyl and a primordial root or the radicle are present in the embryo. The root end of the embryo usually has a root cap.

The zygote most frequently divides transversely.[3] With the division of the two resulting cells, the orientation of the two

new walls may vary. The cell oriented toward the micropyle, the proximal cell, divides transversely. The distal cell may divide transversely, vertically or obliquely. The subsequent divisions are usually distributed unequally in the various tiers of the four-celled embryo. Furthermore, the divisions become specificially oriented in the different tiers resulting in the differentiation of the embryo into the main body or embryo proper, and the suspensor, a filament of cells. Before this differentiation takes place, the young embryo is called proembryo.

The development of an embryo follows an orderly pattern. The differentiation into a root pole and shoot pole indicates an early establishment of polarity and the difference between the two poles increases through differential divisions and cell enlargement in the subsequent embryogenetic stages.

This study was undertaken to serve as background data on future work that pertains to (a) the means and ways by which the knowledge of the different stages of development may be used to promote or inhibit the growth of the plant, and (b) the study of environmental conditions conducive to the maximum development of the different stages in the embryogeny of the plant.

This study deals with the development of the embryo of two species of *Amaranthus, A. spinosus* and *A. viridis,* from the early divisions of the zygote to the formation of the mature embryo.

Several studies were made on the early embryogenetic stages of certain genera of Amaranthaceae, but comparatively very few were made on *Amaranthus.*

In this studies of certain members of the family Amaranthaceae, Puri and Singh[4] found that the embryo sacs are derived from two megaspores of the same tetrad rather than the products of two different megaspore mother cells. The absence of any intervening cells supports this.

According to Joshi and Kajale[5] the embryonic development of Amaranthaceae commences with the usual cell division taking place in most angiosperms until a proembryo, with varying number of cells, is formed. Amaranthaceae may have a long proembryo in which three apical cells take part in the development of the embryo proper. Accessory cells take part in the development of the hypocotyl and the root region of the embryo.

Further studies made in other species by Kajale[6] corroborate with the previous ones, but with varying number of cells in the proembryo. The mature embryo may be annular in form as in *Celosia argentea, Amaranthus viridis, Alternanthera sessilis* and *Digera arvensis.*

Woodcock[7] says that a somewhat curved embryo characteristic of the embryo of *Amaranthus caudatus* is brought about by the elongation of the embryo sac extending to the chalazal region.

Joshi and Kajale[5] stated that embryo development of Amaranthaceae corresponds to the Chenopodiad type. In this type the zygote divides transversely resulting in two cells. The micropylar cell divides and a row of three cells is formed. By further transverse divisions a proembryo of six to seven cells is organized. Bakshi[8] named three species that have this type of embryo development—*Amaranthus retroflexus, Alternanthera sessilis,* and *Digera arvensis.* Since the embryogeny of *Psilostachys sericea* resembles that of these species, he concluded that *P. sericea* belongs to the Chenopodiad type. Padhye[9] however, doubted this conclusion. Johansen[10] indicated that *Amaranthus retroflexus* embryonomically represents in transition form between the Solanad and Chenopodiad types, but since the basal cell of the two-celled proembryo contributes to the formation of the embryo it belongs to the latter type. *Amaranthus caudatus* is identical in development with *A. retroflexus* and *Digera arvensis; Alternanthera sessilis* and *Achyranthes aspera* are also similar. Kajale[11] confirmed this, stating that embryo development in *Achyranthes* does not differ in essential points from that of *Alternanthera* and *Digera* and corresponds to the Chenopodiad type. Soueges[12] gave a comprehensive account of the development of the embryo in *Amaranthus retroflexus* emphasizing the origin of the different layers of the embryo which depends very much on the type of proembryo it has.

Padhye[9] disagreed with the conclusion that most species of this family follow the Chenopodiad pattern of embryo development. One species he particularly observed was Gomphrena celosiodides, which exhibits the Solanad type of embryo development. On the other hand, Davis[13] stressed that embryogeny in Amaranthaceae follows either the Chenopodiad or Solanad type.

Certain unusual phenomena occur in Amaranthaceae. In *Aerva tomentosa*, Sachar and Murgai[14] noted that the cell is capable of developing parthenogenetically. In *Pupalia lappacea*, Kajale[15] reported that the three antipodals may multiply and give rise from thirty to forty cells. During the secondary elongation of the embryo sac, the antipodals are pushed laterally on one side of the embryo sac, and persist after fertilization up to the early stages of embryo development. Davis[13] added another unusual characteristic, polyembryony, in *Celosia cristata* and *Gomphrena decumbens*.

MATERIALS AND METHODS

Flowers from two species of *Amaranthus*, *A. spinosus* and *A. viridis* in various stages of development were collected from residential sections in Paco and Quezon City.

The flowers were fixed in Formo-Aceto-Alcohol (FAA) for at least 24 hours. Specimens were dehydrated through the tertiary butyl alcohol series, and then embedded in paraffin, and cut in longitudinal sections with a rotary microtome at 10 to 11 microns thick. The slides were stained in Safranin-Fast Green combination and mounted in balsam.[16] Mounted specimens were examined individually with a compound research microscope. Illustrations of the specimens were drawn with the aid of the camera lucida. Photomicrographs were also taken.

OBSERVATIONS

Description of the species — The Amaranths are mostly garden weed herbs though some are well-known garden plants of food value.

In the Philippines four taxa occur: *Amaranthus hybridis*, *A. spinosus*, *A. tricolor*, *A. viridis*.[17] The taxa, *Amaranthus spinosus* and *A. viridis*, used in this study are described below.

Amaranthus spinosus locally known as "urai", the young leaves of which may serve as spinach,[18] is found as a weed throughout the Philippines in open waste places, being gregarious and abundant on the roadside and along margins of streams. "Urai" is a monoecious, stout, erect, smooth annual herb 0.4 to 1 m high with slender axillary spines. The leaves are long-petioled, oblong to oblong-ovate or elliptic-lanceolate, obtuse 4 to 10 cm long. The yellowish green flowers are very numerous, stalkness, 1 mm long in axillary clusters and in elon-

gated terminal and axillary spikes. The upper ones are staminate forming globular clusters in leaf axils while the lower ones are pistillate. The bracts are linear, bristles-pointed and as long as the five sepals are longer. Those bracts are ovate to linear, often aristate. There are five stamens. The ovary is superior and compressed, the ovule is unicellular. The fruit is a utricle, wrinkled and nearly as long as the sepals. The seeds are minute, black and shining.[19]

Amaranthus viridis, very similar to *A. spinosus* and locally known as "kulitis" is an erect, smooth unarmed herb, growing from 30 to 60 cm high. This herb differs from *A. spinosus* in the absence of spices on the stems. The inflorescence is terminal and axillary, simple or panicled, interrupted spikes, few or several, the lateral ascending, not much shorter than the terminal forming a panicle 1 to 2 mm long. The flowers are very small, densely disposed, green or brown about 1 mm long. The three sepals of the flowers are oblanceolate, acute and shorter than the fruit. The staminate flowers have three stamens. The bracts are much shorter than the flowers. The leaves are broadly ovate or rhombic ovate, four to ten cm long often retuse, acute or rounded at the base. The fruit is compressed, an ovoid utricle, thin-walled rugose, equalling or exceeding the calyx. The seeds are about 1 mm broad, black or brown.[19] The tender ends including both leaves and stems of "kulitis" are used as leafy vegetables.[18]

Development of the embryo.—In this study, no stages from the first divisions of the fertilized egg to the filamentous stage were obtained. From the material available the earliest stage obtained was a club-shaped structure (Figs. 1 and 27). With the development of the embryo, a spherical structure results with the suspensor clearly visible (Fig. 2). Further divisions of the embryo proper gives it a top-shaped form with accompanying increase in size (Fig. 3). The top-shaped embryo soon changes into heart-shape as the cotyledon primordia are initiated as shown in Figs. 4, 5, and 28. Subsequent elongation of the cotyledons and the exis of the embryo resulted in a torpedo-shaped structure (Figs. 6, 7, and 29). A small notch can be seen in between the cotyledons. With continued growth the embryo tends to curve as it matures (Figs. 8 and 30). The suspensor is not visible anymore at this stage.

Table 1 indicates the relative increase in size of the embryo as development takes place.

TABLE 1. *Table showing the size of the embryos from the earliest stage (Stage 1) to the mature stage (Stage 8).* +

Stage	B		E				S	
			A		C			
	L	W	L	W	L	W	L	W
1.	40	30	—	—	—	—	15	10
2.	45	40	—	—	—	—	20	10
3.	70	80	—	—	—	—	—	—°
4.	70	60	—	—	—	—	—	—°
5.	—	—	80	100	40	35	15	20
6.	—	—	120	110	90	40	40	30
7.	—	—	130	120	100	40	50	30
8.	—	—	800	130	700	70	—	—°

+ Measurements given in microns.
Legend:
B = Body of the embryo
E = Embryo proper
A = Axis
C = Cotyledons
S = Suspensor
° No suspensor visible.

At its early developmental stage, the embryo consists of relatively unspecialized cells which are more or less alike and are still at a low level of differentiation. The multicellular embryo is curved, with a shoot apex at one end and a root apex at the other.

Establishment of organs.—The early embryogenetic stages of *A. spinosus* and *A. viridis* show an undifferentiated mass of cells. However, the suspensor can be distinguished even at an early stage. In Fig. 2, the lower part of the embryo shows the short suspensor. In this spherical stage, the suspensor can be recognized as composed of vacuolated cells with or without nuclei (Fig. 15). The suspensor reaches the height of its growth at the stage shown in Figs. 6 and 7.

There is an early establishment of polarity as the embryo differentiates into a root pole and a shoot pole. This can be seen already in Fig. 1. The difference between the two poles increases through cell division and cell enlargement in the successive stages of embryogeny. The distal end of the embryo expands laterally on either side as a result of the initiation of the cotyledons (Figs. 4 and 28). The sides of the embryo from

where the cotyledons will arise, start to undergo various cell divisions. The outermost layer is composed of regularly shaped cells which have undergone anticlinal divisions (Figs. 9 and 28). Beneath this layer of cells there is an enlargment of cells accompanied by more anticlinal divisions.

When the cotyledons emerge as a result of cell divisions, both anticlinal and periclinal, the cotyledonary lobes are formed (Fig. 17b). The outer layer of cells retain their shape while the inner cells assume different shapes (Figs. 10 and 11) Figs. 12 and 19b show a longitudinal section of the upper portion of a cotyledon of a mature embryo. The innermost cells are elongate and narrow as compared to those in Fig. 11 and the cotyledons have narrowed down (Fig. 12). The detailed structure of a portion of the cotyledons (Fig. 13) shows that there are various shapes and sizes of cells making up the cotyledons of the mature embryo. The outermost layer is made up of rectangular and regularly shaped cells. The layers below it are made up of large cells, some spherical, some elongate and some irregularly shaped. The innermost cells are very small and irregular differentiating them from the surrounding cells. Intercellular spaces are also formed as a result of the change in size and shape of the cells.

The hypocotyl-root axis has the two cotyledons and the shoot apical meristem at its upper end, while the root primordium covered by the root cap is at its lower end. From the heart-shaped stage (Fig. 17a), cells start to differentiate, leading to the formation of this hypocotyl-root axis. The middle cells elongate and are surrounded by larger cells. In the torpedo-shaped structure, continuous divisions in various directions and the meristematic activity at the shoot and root apices produce more cells causing the main axis to elongate and enlarge (Fig. 18). Cells enlarge and assume characteristic shapes not found in previous stages. The mature embryo is made up of peripheral rectangular cells, small spherical and elongate cells toward the shoot apex and long, narrow cells near the root apex. A portion of this mature embryo stage is shown in Fig. 19a.

The part of the apex left between the two cotyledons makes up the shoot apical meristem of the epicotyl (Figs. 5 to 7). The shoot apex arises superficially and starts to take form in the heart-shaped stage (Figs. 17a and 28), when the part between the cotyledonary initials becomes a little elevated due to peri-

clinal and anticlinal divisions of the cells. After the cotyledons have emerged, the single layer of cells of the shoot apex increases in size and undergoes periclinal divisions giving rise to irregularly shaped cells beneath it (Figs. 21a, b). The shoot apex widens in the torpedo stage (Fig. 23) and consists still of a single layer of cells which through continuous cell divisions produces more cells making the apical meristem increase in size. Finally in the mature stage, the shoot apex (Figs. 25 and 31a) has become narrow. There are two cell layers of initials which undergo anticlinal divisions contributing to its surface growth. Periclinal divisions give rise to irregularly shaped cells beneath the peripheral layers of cells. These cells divide periclinally first, followed by divisions in various planes increasing its volume.

The apical meristem of the root is established on the opposite pole. It produces cells not only toward the axis, but also away from it giving rise to the root cap. The root apex differs from the shoot meristem in that it is not terminal but subterminal in position because of the presence of the root cap. Furthermore, the root apex does not form lateral appendages and grows more uniformly in length compared to the shoot apex.

At the time the cotyledonary lobes have already been formed, the root apex does not show much differentiation. There is only an increase in the layers of cells due to cell divisions but the cells do not change much in shape (Figs. 22a, b). In Fig. 24, changes in the cells can already be observed. Through periclinal and anticlinal divisions, small cells are produced toward the axis, while bigger ones give rise to the root cap. In the mature stage (Figs. 26 and 31b) the root meristem with its root cap has increased in size because of the actively dividing and growing cells in this region. The root tip cells divide periclinically and anticlinally giving rise to the various tissues of the root. The root cap has a common origin with the epidermis.

Establishment of tissues.—The continuous divisions of the cells of both the shoot and root apices produce new cells which make up the bulk of the early embryo. It is still relatively simple and homogenous (Figs. 14 and 27). Early differentiation gives rise to an embryo consisting of three primary meristems: the protoderm, which is the outermost layer, the procambium in the center and the ground meristem between the protoderm and pro-

cambium.The protoderm which gives rise to the epidermis can already be distinguished from the spherical stage. It does not extend to the suspensor but fuses with the apical meristem of the root (Fig. 15). It is usually the first of the tissue systems to be formed consisting of regularly shaped cells. The top-shaped structure (Fig. 16) shows characteristics typical of the protoderm—rectangularly-shaped cells, uniform in size and shape. However, in the heart-shaped stage and the subsequent stages until the mature stage (Figs. 17a, 20a, b) the cells show more distinct characteristics. They stain lightly and do not have many vacuoles. Some cells tend to increase in size though most of them are smaller than the inner cells.

The procambial system can be recognized from the heart-shaped stage (Figs. 17a and 28). The innermost cells start to differentiate into long, thin cells characteristic of procambial cells. The cells in this region absorb stain readily due to their dense cytoplasm. They undergo rapid cell division and cell elongation, and extend into the cotyledons in continuity with that of the embryo axis. The mature embryo (Fig. 19a) shows the elongated procambial cells near the root apex. In the cross-section of the hypocotyl and cotyledons, the procambial cells can be recognized as the smallest but most actively dividing cells (Figs. 20a, b and 32a, b).

Enlargement and increased vacuolation of cells surrounding the procambium block out a tissue region, the ground meristem. In the torpedo stage (Fig. 18), the cells enlarge and the nuclei stain deeply. The cells here stain more than the protoderm but lighter than the procambium. In this region, the cells are rectangular and polyhedral. They are larger than those of the protoderm and somewhat loosely arranged and therefore intercellular spaces are formed. Due to cell enlargement without corresponding increase of cytoplasm, the cells become vacuolated. Some cells are completely devoid of cytoplasm (Fig. 19a).

Figs. 20a, b and 32a, b show how the different primary meristems are clearly delimited. The outermost layer with regularly shaped cells is the protoderm. The layer of cells beneath this is composed of irregularly shaped cells—the ground meristem. The innermost cells, the smallest ones, make up the procambium. These layers—the protoderm, procambium, and ground meristem will give rise to the epidermis, central cylinder and cortex respectively.

DISCUSSION

Attempts have been made to follow the development of the embryo from its earliest stage to the later stages in the family Amaranthaceae.[7,12] In *Amaranthus retroflexus* and *A. viridis*, the fertilized egg divides transversely to produce an apical cell and a basal cell.[6,12] A transverse division of the apical cell then results in a second basal cell adjacent to the first. Further transverse and longitudinal divisions form the proembryo which consists of a filament of cells.[6] Soueges[12] discussed in detail the different modes of development of the zygote of *Amaranthus retroflexus* and *A. caudatus* from its first division to the formation of the three primary meristems—the dermatogen, periblem and plerome. Woodcock[7] touched a little on the embryo development of *A. caudatus* but his main interest was on the development of the seed. Kajale[6] compared six species representing six genera of Amaranthaceae. He included microsporogenesis, megasporogenesis and embryo development until the perisperm and endosperm are formed.

Much of what has been written on Amaranthaceae deals with the early stages of embryogenesis with emphasis on the origin of tissues from the early formed cells, and the formation of the perisperm and endosperm. The present study deals more with the later stages of embryogenesis of *A. spinosus* and *A. viridis*. The planes of division and the increase in the number and size of cells found in these two species are like those of the species that have been reported earlier by other workers. Soueges[12] observed that in *A. retroflexus* and *A. caudatus*, the final formation of the primary meristems, like the dermatogen formed from the outer layer of cells, the periblem and plerome from the inner layers, are differentiated from the top-shaped embryo. The beginnings of the hypocotyl, root and cotyledons are also pointed out. *A spinosus* and *A. viridis* show tissue differentiation of the embryo as early as the spherical stage (Fig. 15). In the heart-shaped stage, there is more differentiation of cells especially at the sides where the cotyledons originate and the middle portion where the procambium forms. Cotyledonary development is seen first as two bumps that gradually increase in length and width until they have reached their maximum size in the mature embryo.

In his comparative study of the six species representative of Amaranthaceae, Kajale[6] discussed *A. viridis* as one of them.

He showed embryo development of *A. viridis* from fertilization until the formation of the mature embryo, but did not go into much detail.

In his study of *A. caudatus*, Weedcock[7] described the proembryo as being differentiated into the embryo proper and the many-celled suspensor. He mentioned the cotyledons and plumule in connection with the cellular endosperm, stating that the endosperm which at first is free nuclear, becomes cellular when the cotyledon and plumule primordia begin to appear. He described the radicle, hypocotyl and the two cotyledons as semicircular in cross-section. The mature embryo is curved and in contact with the testa. Almost half of the embryo consists of cotyledons. In *A. spinosus* and *A. viridis*, the cotyledons also make up almost half of the mature embryo. In these cotyledons, the three layers, the protoderm, ground meristem and procambium can be distinguished.

SUMMARY

The development of the embryos of *Amaranthus spinosus* and *A. viridis* was studied in successive stages from the club-shaped stage to the mature embryo. Sections of the early stages of development of the embryo show the club-shaped structure having a mass of undifferentiated cells; a spherical stage with the suspensor still visible, and a top-shaped structure with the outer layer differentiated. As the embryo takes on a heart-shaped structure, the cotyledonary initials appear on both sides. These cotyledonary initials develop into cotyledonary lobes elongating and enlarging until they reach their maximum length in the mature embryo. At the same time the hypocotyl-root axis develops from the heart-shaped stage to the torpedo stage. The hypocotyl at this stage is made up of large cells beneath the peripheral layer and elongated cells in the middle portion. In the mature embryo stage, there are several small spherical cells near the shoot apex while long and narrow cells occur near the root apex.

Even in the embryogenic state, primary meristems are already formed. There are three primary meristems in the embryo of *Amaranthus spinosus* and *A. viridis* with the protoderm as the first one formed. It consists of a layer of regularly shaped cells recognizable from the spherical stage. The procambium, the innermost layer, is the next one formed. It is

made up of small, narrow, long cells usually actively dividing and filled with densely staining cytoplasm. The formation starts in the heart-shaped stage. Finally the ground tissue meristem is recognized as a tissue region blocked out from the other layers by its enlarged, irregular cells and their increased vacuolation. This takes place during the torpedo stage. In the mature embryo, these regions can be recognized by the characteristics particular to each of them.

In order that a study like this can be useful, it is necessary to implement the knowledge provided by continued experimental research, which can help obtain a better grasp of the phenomena taking place in the developmental process of the embryos of these plants, *Amaranthus spinosus* and *A. viridis*.

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EXPLANATION OF FIGURES

Fig. 1. Club-shaped embryo. x 100.
Fig. 2. Spherical embryo with suspensor. x 100.
Fig. 3. Top-shaped embryo. x 100.
Figs. 4-5. Heart-shaped embryos. x 100.
Figs. 6-7. Torpedo-shaped embryos with suspensor. x 100.
Fig. 8. Mature embryo with two cotyledons. x 100.
Fig. 9. Cotyledonary initials in heart-shaped structure. x 450.
Fig. 10. Portion of cotyledon in heart-shaped structure. x 450.
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Fig. 12. Tip of cotyledon in mature embryo. x 100.
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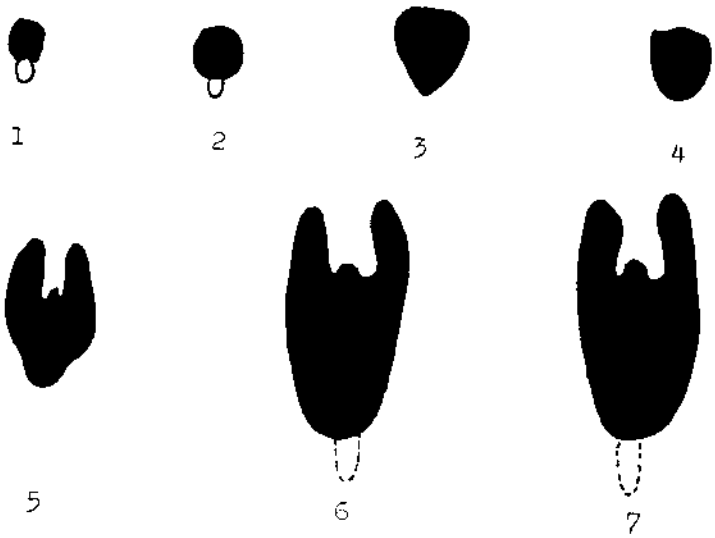
N.B. All drawings were made with the aid of the Camera Lucida.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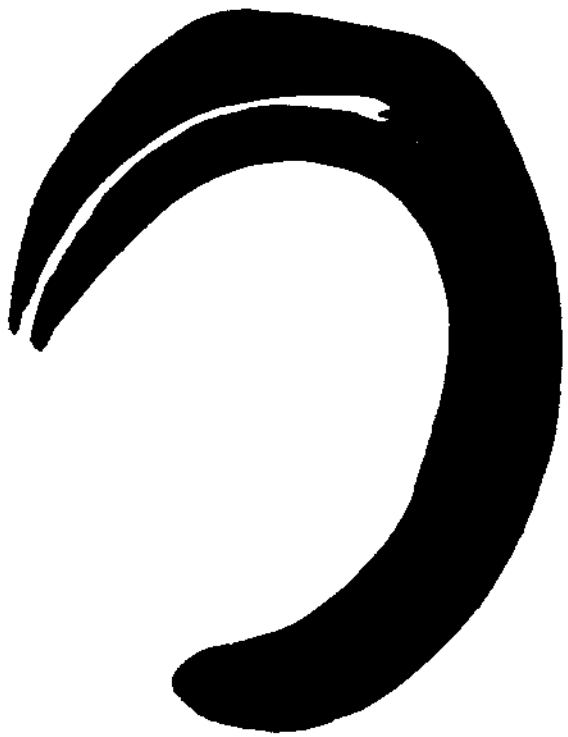
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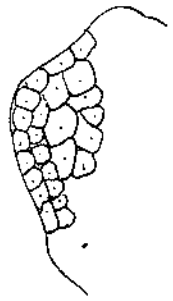

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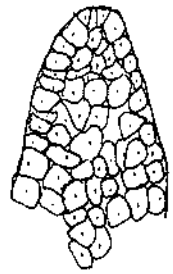

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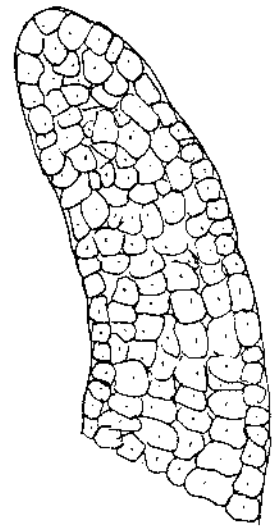

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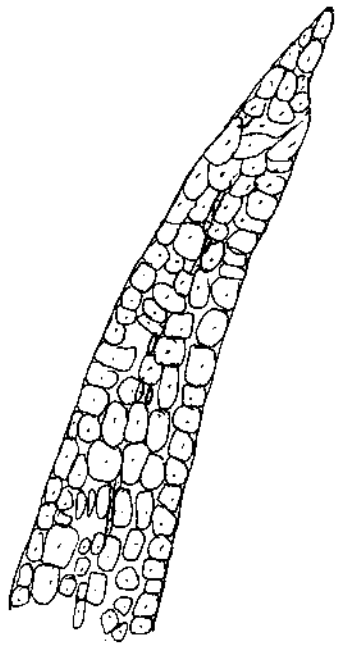

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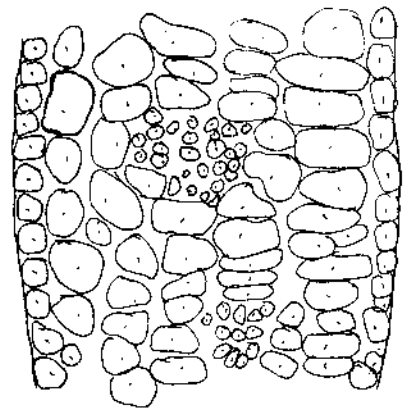

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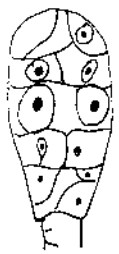

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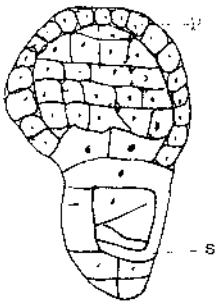

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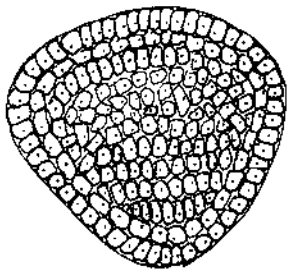

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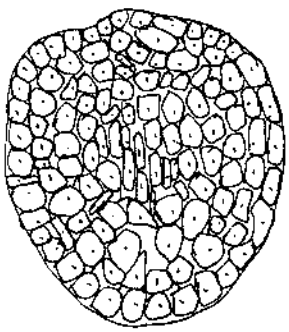

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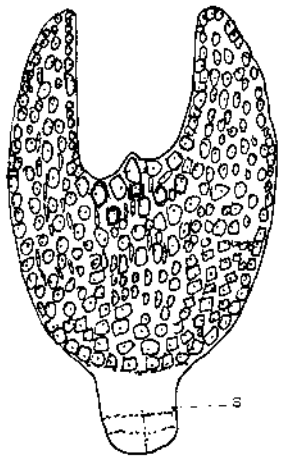

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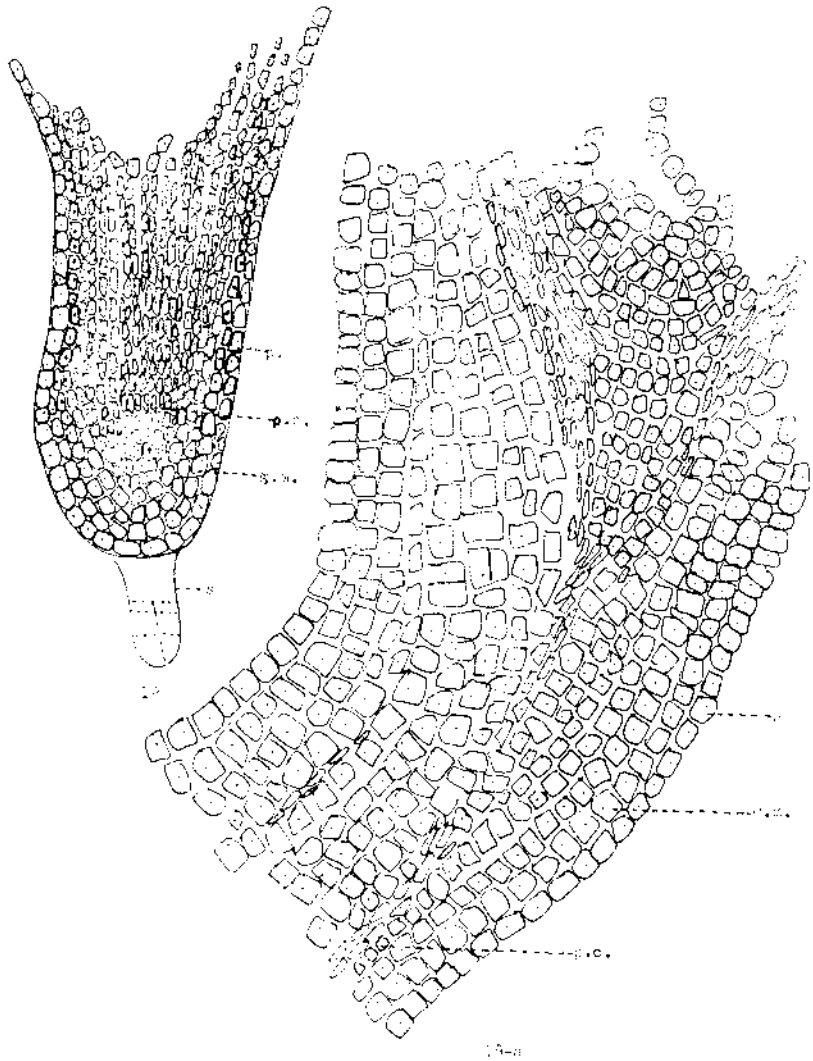

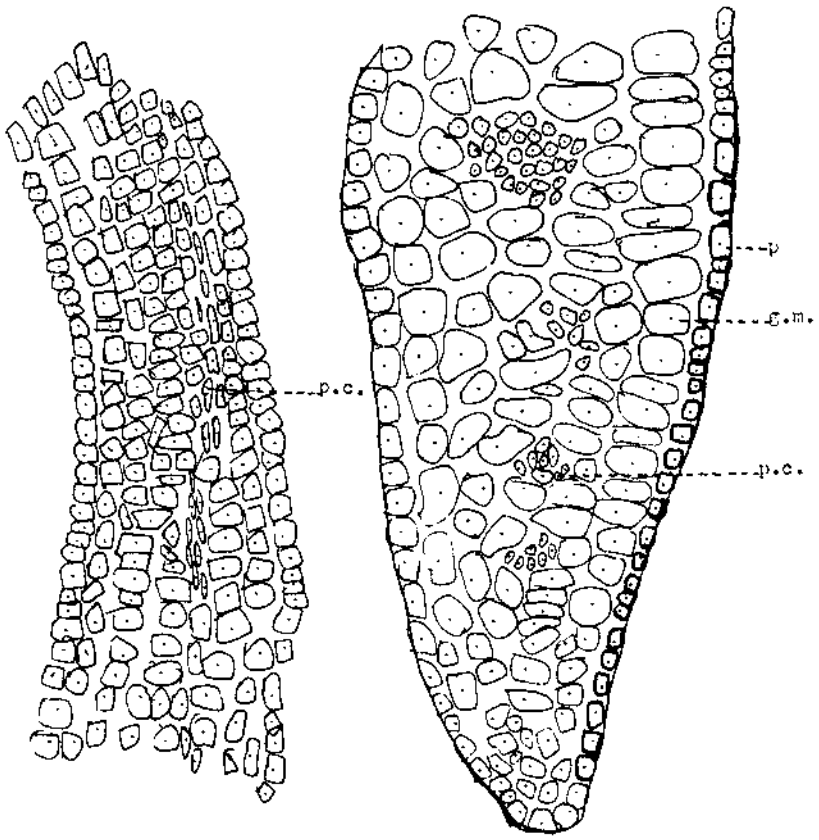

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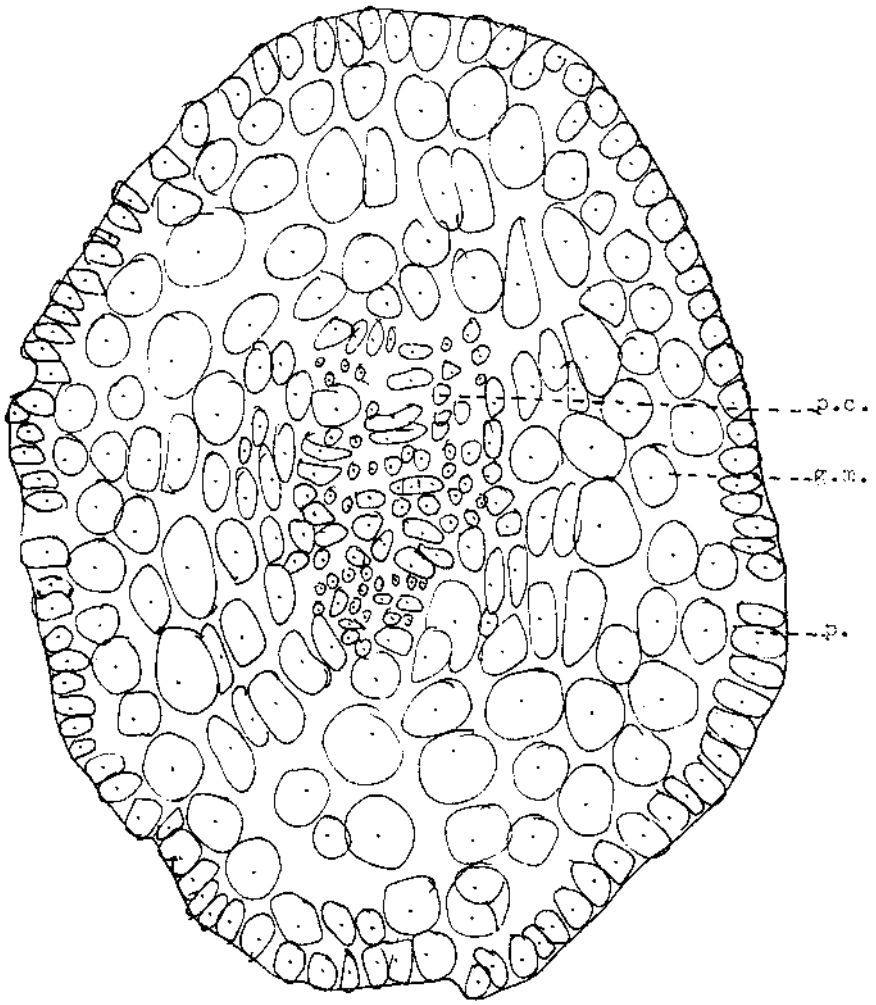

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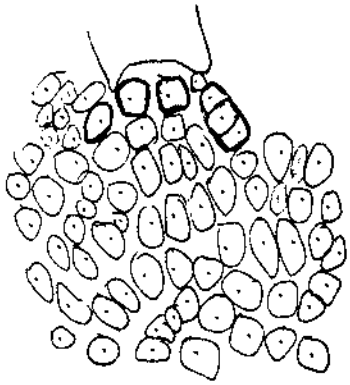

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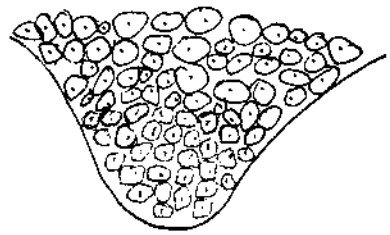

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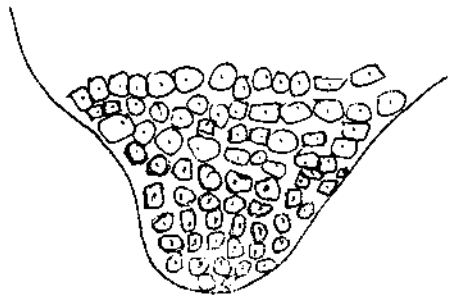

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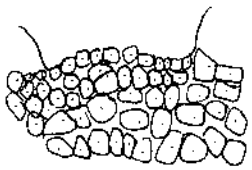

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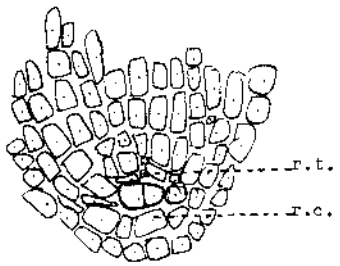

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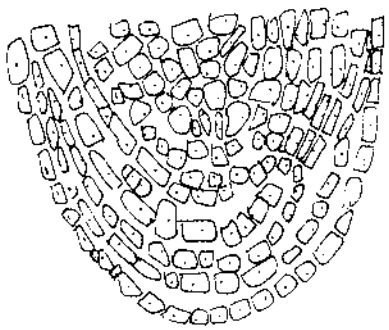

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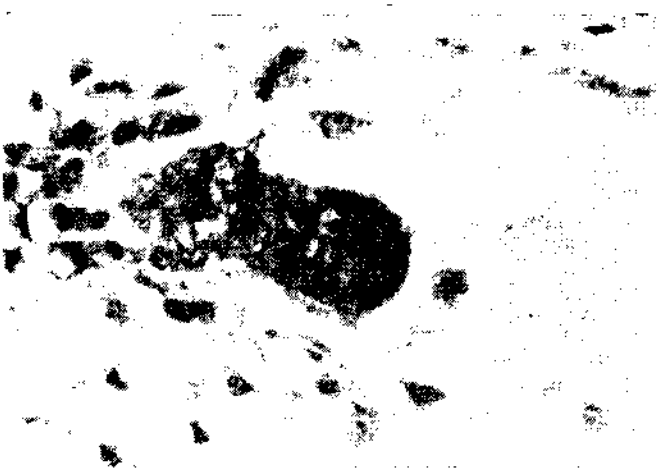

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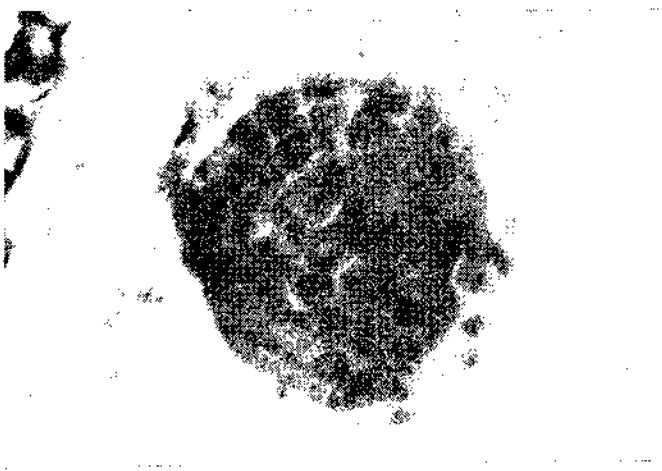

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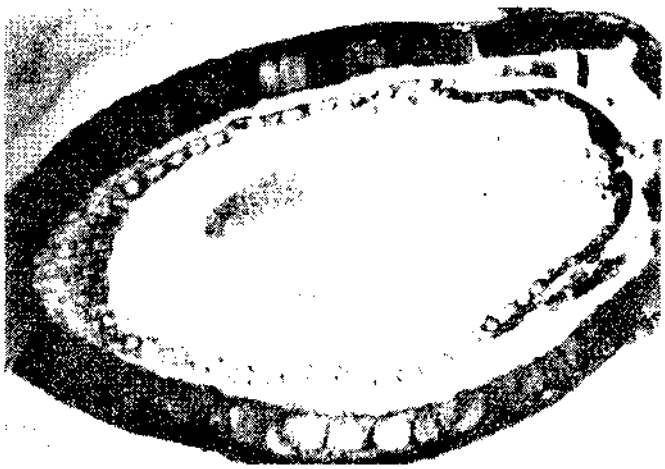

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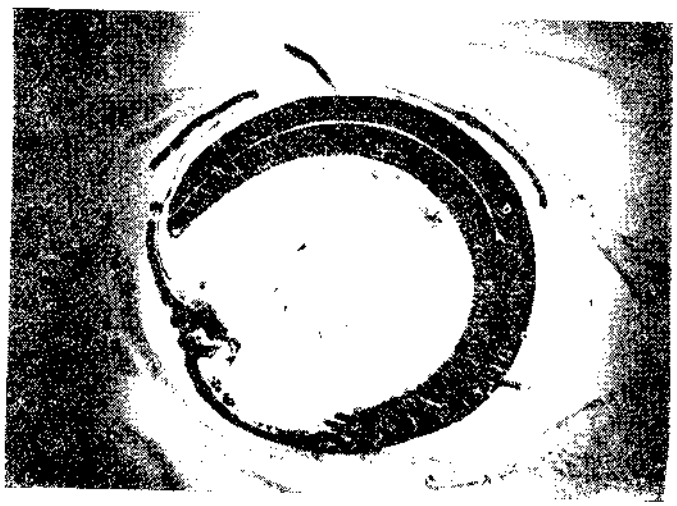

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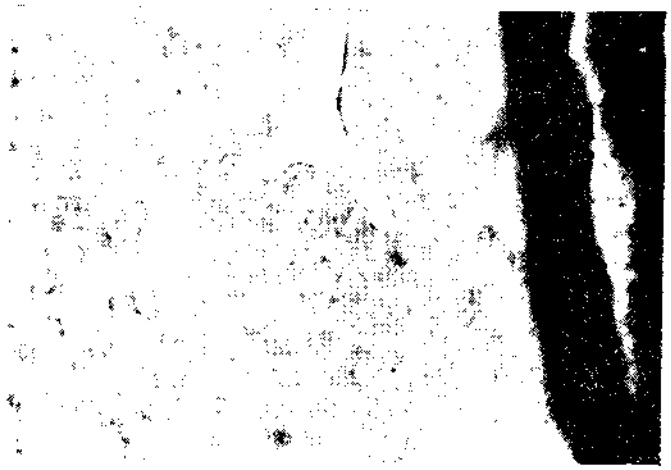

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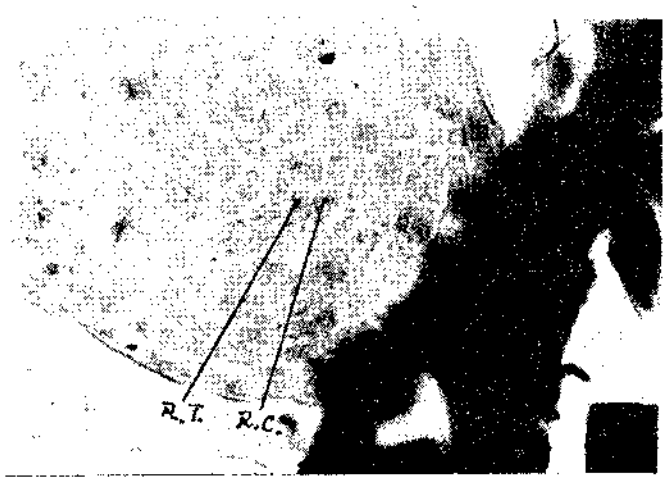

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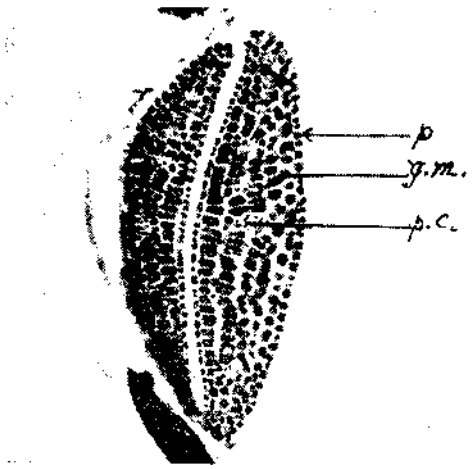

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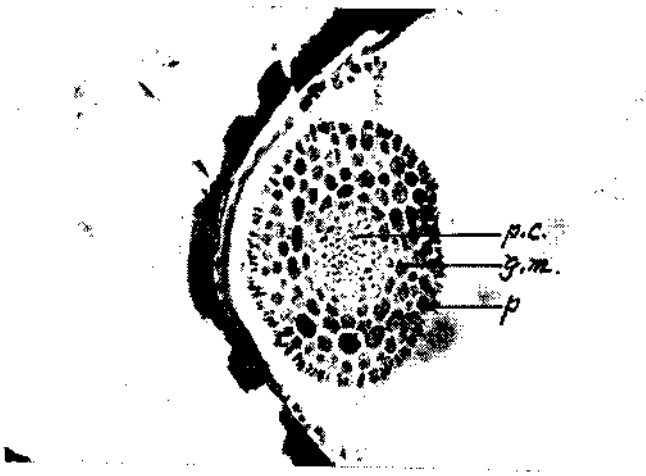

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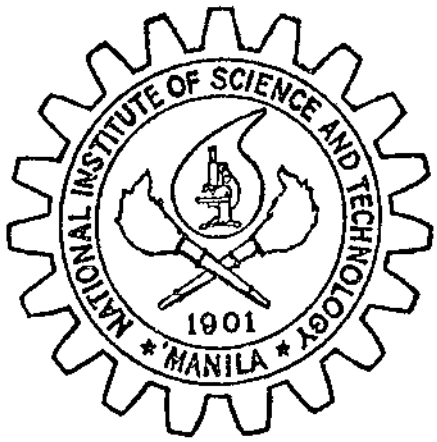

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